Development of Gradient Stationary Phase Optimized Selectivity Approaches for Improved Method Development in High Performance Liquid Chromatography

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Chapter 1 Introduction, aims and outline

High Performance Liquid Chromatography (HPLC) is after the mass balance and the pH meter the most frequently used analytical technique worldwide [1]–[4]. It is intensively applied in for instance the life sciences, pharmaceutical, fine chemicals and the food industry for separating a versatile amount of compounds in a broad variety of samples. Depending on the physicochemical characteristics of analytes such as the polarity, molecular weight, hydrophobicity, charge, etc., multiple modes of liquid chromatography (LC) with each having a unique separation mechanism can be applied. Initially, LC was developed as a normal phase technique [5], [6] and was later on, from the 1970s on, quickly and increasingly replaced by Reversed Phase LC (RP-LC) as the most suitable LC mode for the majority of applications [7]–[11]. NP-LC is appropriate for the separation of compounds by polarity, while RP-LC is adequate for the separation of compounds based on hydrophobicity. In the last two decades, HILIC (Hydrophilic Interaction Liquid Chromatography) has gained a growing interest and importance as an additional separation mode as it shows a high potential for the analysis of hydrophilic compounds which are not soluble in NP-LC solvents [12]–[15]. Not to be confused with HILIC, the aqueous normal phase LC mode (ANP-LC) [16] offers new avenues for the separation of both polar and non-polar compounds by using silica-hydride-based stationary phases and whereby varying the amount of water in the mobile phase, all silica-hydride-based separation materials can operate in the reversed-phase mode, the normal-phase mode or, in some instances, a combination of both. Other well-known modes of HPLC are Size Exclusion Chromatography (SEC) where separation occurs based on molecular size [17] and Ion Exchange Chromatography (IEC) which can be used for the separation of ionic and ionziable compounds [18], [19]. Concerning the separation of enantiomers, the term Chiral HPLC is often used [20].

In this work, emphasis is set on RP-LC as it is today the main separation mode for the analysis of organic molecules. RP-LC is a core technique in the pharmaceutical industry for e.g. the separation of an active pharmaceutical Ingredient (API) from its related compounds. The latter compounds can be impurities which can be present in a drug substance (DS) batch as such, or degradation products or preservatives such as parabens or other excipients. Often, a complete separation is requested to be able to identify and to accurately quantify all compounds in a sample. For example, regarding the purity of pharmaceutical products, regulatory authorities set specifications and demand the monitoring of all the present known and unknown impurities. For known impurities, specification levels might be more tolerant depending on their level of toxicity. A specific example application

hereby is the analytical follow-up during stability studies of pharmaceutical products [21], [22]. The assay of the API and the impurities should be monitored as a function of time, as well as the degradation products which may appear during the stability study. In order to obtain such RP-LC separations which allow complete baseline separation, suitable methods have to be developed. A suitable method should offer specificity for each relevant analyte and thus, overlapping peaks are unwanted. As a consequence, the aim of an acceptable resolution for peak pairs is a crucial part in pharmaceutical method development [21], [22].

But RP-LC does not play an important role in the pharmaceutical industry only. It is by far the technique of choice for the analysis of biological and natural compounds such as alkaloids [23], steroids [24] or vitamins [25]. Other RP-LC separation applications are situated in the food industry for the analysis of e.g. sweeteners [26] or in environmental analysis for the analysis of e.g. pesticides [27].

In the past years, much focus has been set on efficiency enhancements for improving the resolution of peak pairs, somewhat to the detriment of selectivity optimization. By contrast, in this work the focus is set on selectivity as an essential tool for improving or rather optimizing the resolution of peak pairs. In other words, the aim of this thesis is to deepen out selectivity optimization in RP-LC and to extend the possibilities for tuning and optimizing the selectivity beyond the current applied optimization approaches.

In a first part of this work (Chapter 2), an overview is presented about the contemporary approaches which are used in RP-LC selectivity optimization. Therefore, Chapter 2 is started with a fundamental explanation of the fundamental chromatographic parameters and their influence on selectivity. The most relevant practical issues of HPLC of relevance for the work are outlined and subsequently some recent potent developments are described.

Subsequently, focus is set on the stationary phase as a tool to optimize selectivity in a rational way. This approach is called stationary phase optimized selectivity liquid chromatography (SOSLC). This involves an empiric strategy whereby the optimization output results in an optimized serial sequence of different stationary phases which are linked to each other into one SOSLC application specific column. The actual parameter to be optimized is thereby the partial column length of each individual type of stationary phase used in this strategy. The advantages and disadvantages of this strategy are first assessed and extended solutions are subsequently presented to overcome the observed limitations.

For complex mixtures containing compounds which encompass a broad range of hydrophobicity, isocratic elution is often elusive as the mobile phase composition and thus the eluotropic force are invariable. The possibility of a multistep-gradient approach in SOSLC is investigated to overcome this limitation (Chapter 3). This approach is based on a rather rough but fit-for-purpose strategy and is demonstrated with the separation of a mixture of 27 steroid compounds. The compounds are thereby first subdivided into three groups according to their relative hydrophobicity. For each group an adequate isocratic mobile phase composition level is selected for which isocratic SOSLC is performed with a set of five different stationary phases. The optimization output results in an SOSLC column which offers sufficient selectivity for the compounds of all three groups and whereby a multistep-gradient of the three selected mobile phase composition levels is applied.

Subsequently, an extension to linear gradient SOSLC is developed and described in Chapter 4. With this extension and in contrary with the rough multistep-gradient approach, an algorithm has been developed for the accurate prediction of retention times under linear gradient conditions and which is applicable to a column consisting of different serially coupled stationary phase segments. The development of the prediction algorithm was evaluated by means of a test mixture of several small organic molecules. Subsequently, linear gradient SOSLC has been evaluated with a set of five different stationary phases and a steroid model mixture for which a baseline separation is obtained on an optimized SOSLC column with a generic linear gradient.

Further on and focused on SOSLC as a driving force for selectivity optimization, the utility of SOSLC is investigated with the more inconvenient but green organic modifier ethanol (Chapter 5). This chapter fits in a growing tendency where efforts are made for replacing toxic solvents by *green* solvents. Beside, this creates opportunities where classic solvents may be subjected to price fluctuations. This investigation is performed by means of a model mixture containing several compounds of different types of small organic molecules.

Finally, the SOSLC principle is fitted within the increased efficiency approach whereby an extended column length is aimed at in combination with elevated temperatures (Chapter 6). In this way, benefits of increased efficiency are combined with the selectivity enhancement offered by the SOSLC strategy. The increased efficiency is demonstrated by means of two model mixtures, offering a solution to typical difficult separations of a pharmaceutical compound from its impurities. Hereby the degree of separation is not only assessed by the selectivity but also by means of a discrimination factor which takes in account the tailing of a typical broad API peak.

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Chapter 2 Fundamentals of selectivity optimization in HPLC

2.1 Introduction

High Performance Liquid Chromatography (HPLC) is a physical separation technique based on the partitioning of components of a sample between a mobile phase and a stationary phase. LC can be broadly subdivided according to the physical orientation of the stationary phase, which can be positioned in a cylindrical column or on a planar carrier as in thin layer chromatography (TLC). In a cylindrical column, the stationary phase can be positioned on the wall of the column (open tubular LC) or on porous or semi-porous particles which are packed in the column. Packed column HPLC is nowadays by far the most used LC technique as packed columns offer advantages such as reproducibility, durability, sample capacity and phase selectivity options [1]. Detection subsequently occurs by means of an online detector which monitors the column effluent and generates a signal for the eluting separated components. This results in the generation of a chromatogram. As in this work only packed column HPLC was performed, emphasis will only be set on this technique.

2.2 Fundamental concepts of HPLC

The three fundamental factors of a chromatographic separation are retention, selectivity and efficiency. As each of these factors has a tremendous influence on the chromatogram that is obtained, it is essential to explain and define these factors in detail before outlining how they can be used in state-of-the-art applications [2], [3].

2.2.1 Retention and the retention factor

Figure 2.1 shows a typical chromatogram. A detector response is thereby plotted versus the time on the axes. Further, an injection peak and two analyte peaks are included. The time between the moment of injection, which corresponds to zero on the time axis, and an analyte reaching the detector is called the retention time t_r [2], [3]. The retention time of what would be an unretained component and which is often visible by the first baseline disturbance caused by the elution of the solvent, is termed the void time t_0 or also called the injection peak.

A peak, which has ideally a Gaussian shape, is characterized by a width W_b and a height h. Sometimes, the width halfway up the peak $W_{0.5}$ is used to meet aspects such as column performance

[2]–[4]. For some calculations, the standard deviation σ_t of the peak is required. This standard deviation is equal to half the peak width at 60.7% of the peak height. The height or the area of a peak corresponds to the amount or concentration of that particular component in the sample. Both parameters can be used to perform quantitative analysis. However, the first is usually preferred since it provides more accurate quantitative measurement [4].

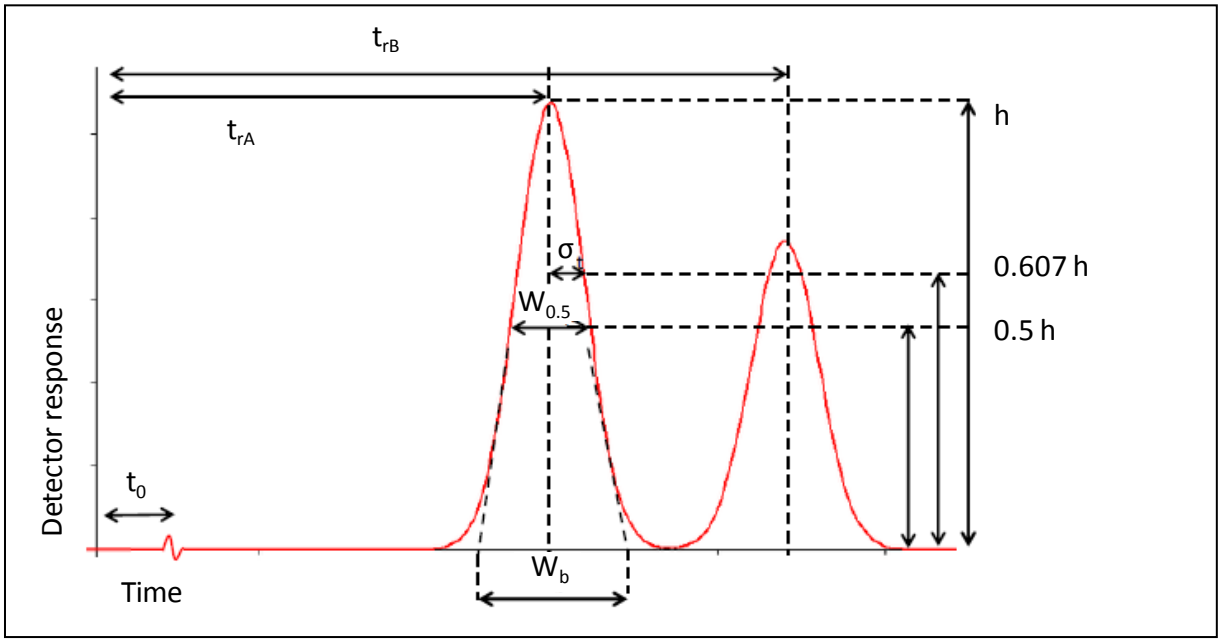

Fig. 2.1 Representative chromatogram displaying the analysis of two separated peaks and representing the dead time (t_0), the retention time of peaks A (t_{rA}) and B (t_{rB}), the peak height of peak A (h) and the different variables used to express the width of peak A: the peak width at the base (W_b), peak width at half height ($W_{0.5}$) and the standard deviation of the peak (σ_t).

While retention time is used for peak identification, it is dependent on the applied flow rate, the column dimension and on other parameters. A more fundamental term that measures the degree of retention of the analyte is the retention factor k as the ratio of the net retention time t_r' – retention time t_r minus the void time t_0 – by the void time [2]–[4]:

$$k = \frac{t_r - t_0}{t_0} = \frac{t_r'}{t_0} = \frac{K}{\beta} \qquad \text{(eq. 2.1)}$$

This factor measures how much the analyte is retained compared to an unretained analyte [5]. A value of 0 for k means that the analyte is not retained and elutes at the void time. As a rule of thumb, values for k are preferably between 3 and 20 as this results in adequate retention and sufficient time for the analyte to interact with the stationary phase. This allows differential migration of the present analytes through the column, resulting in separation of these analytes. Analytes eluting at a high value of k are often problematic due to e.g. excessive analysis times or poor sensitivity as a result of excessive peak broadening in the column in the case of isocratic analysis. Equation 2.1 also shows the

link of the retention factor with the partition coefficient K and the phase ratio β. K thereby represents the ratio of the concentration of the analyte in the stationary phase and in the mobile phase. The phase ratio β corresponds to the ratio between the volume of mobile phase and stationary phase [2]–[4].

2.2.2 Selectivity

As mentioned earlier, separation between two analytes occurs if they depict different velocities through the column. The term selectivity touches on the degree of difference in retention and thus on the separation for two analytes and is expressed mathematically by means of the selectivity factor α [2], [3]:

$$\alpha = \frac{t_{r'B}}{t_{r'A}} = \frac{k_B}{k_A} = \quad \text{(eq. 2.2)}$$

Hereby, the selectivity factor α of a pair of peaks is the ratio of the retention factors of the later eluting peak over the earlier eluting analyte. As a consequence, the selectivity factor is always larger than 1 (equal to 1 in the case of overlapping analytes). The concept of selectivity is visualized in Figure 2.2. The focus in this work is set on selectivity.

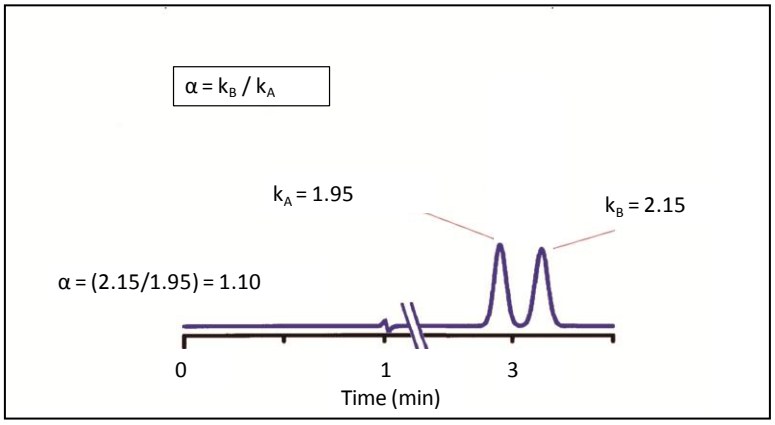

Fig. 2.2 Chromatogram of two closely eluting peaks with a selectivity factor of 1.10.

2.2.3 Efficiency

As can be observed in most chromatograms, peaks tend to have a Gaussian shape. Besides, they seem to broaden with time under isocratic conditions whereby the width becomes larger with longer retention times. This is caused by several brand-broadening effects that take place inside the column. Hereby, the term efficiency touches on the width of a peak and is mathematically expressed by means of the plate number N [2], [3].

The plate number N, which is a measure for the efficiency of the column, is defined as the ratio of the retention time t_r over the standard deviation of the peak width σ_t [2], [3]. For Gaussian peak shapes, the following equation is valid [2], [3]:

$$N = \left(\frac{t_r}{\sigma_t}\right)^2 = 16\left(\frac{t_r}{W_b}\right)^2 = 5.54\left(\frac{t_r}{W_{0.5}}\right)^2 \quad \text{(eq. 2.3)}$$

The concept of plates is traditionally derived from the distillation process, whereby a longer distillation column would have more "plates" or equilibration steps. Similarly in chromatography, the height equivalent of a theoretical plate (HETP or H) is equal to the length of the column L divided by the number of theoretical plates N even though there are no discrete plates inside an HPLC column [2], [3].

$$H = \frac{L}{N} \quad \text{(eq. 2.4)}$$

In a column packed with fully porous particles, the achievable minimum for H (H_{min}) is twice the particle diameter of the stationary phase d_p [6].

$$H_{min} = 2d_p \quad \text{(eq. 2.5)}$$

Therefore, the theoretically achievable number of plates is:

$$N = \frac{L}{2d_p} \quad \text{(eq. 2.6)}$$

As a result, columns packed with smaller particles are more efficient and allow faster analysis as they can generate a certain amount of plates for a given column length.

In relation with the efficiency, the van Deemter equation links the plate height and the mobile phase linear velocity u. This equation is not the only but the most known and used model to describe the intra-column band broadening [7]:

$$H = A + \frac{B}{u} + Cu \quad \text{(eq. 2.7)}$$

The model assumes that the plate height is composed of three different and independent contributions to band broadening.

The A term represents the physical phenomenon known as Eddy dispersion [8] which contributes to band broadening due to different pathway lengths of the individual molecules of an analyte when

they migrate through a packed column. The A term is proportional to the particle diameter d_p and can mathematically be described as:

$$A = \lambda' d_p \qquad \text{(eq. 2.8)}$$

λ' is a hereby a factor which influences the A term and expresses the quality of the packing. In the van Deemter equation (equation 2.7), the A term is independent of the mobile phase linear velocity u.

The B term represents the longitudinal diffusion and is proportional to the diffusion coefficient D_m of the analyte in the mobile phase. In the van Deemter equation (equation 2.7), the B term is inversely proportional to the mobile phase linear velocity u. Physically, this means when the mobile phase flow is low, the analyte remains for a longer time in the column and hence has more time to diffuse. Therefore, under isocratic conditions the peak broadening will typically be larger for late eluting analytes compared to analytes which elute early. A direct consequence is the decrease of detection sensitivity of such late eluting analytes.

The C term expresses the resistance to the mass transfer of an analyte between the mobile phase (C_M) and the stationary phase (C_S) and is proportional to (d_p^2/D_m). C_M and C_S are assumed to have an additive contribution to the C term. In the van Deemter equation (equation 2.7), the C term is proportional to the mobile phase linear velocity.

The physical phenomena which contribute to band broadening and which are described by means of these three terms in the van Deemter equation are visualized in Figure 2.3.

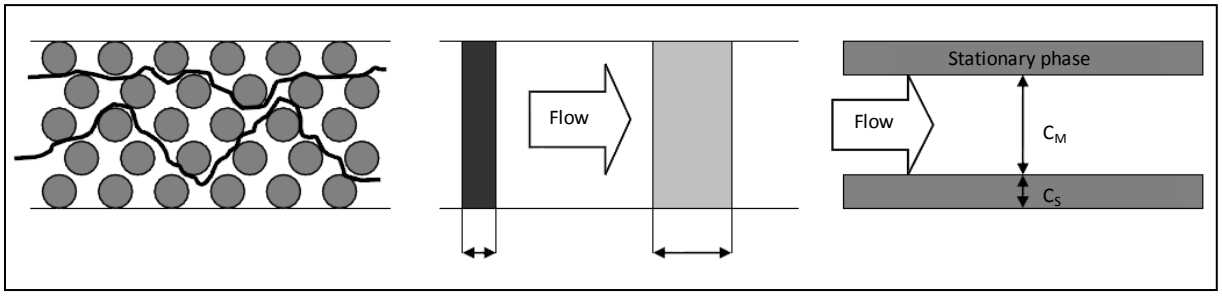

Fig. 2.3 Visualization of the physical meaning of the three terms of the van Deemter equation (equation 2.7). Left: the A term for ddy dispersion. Two random pathways through a packed column are shown. Differences in pathway length leads to peak broadening. Middle: the B term for longitudinal diffusion. During its migration through the column, an analyte band suffers from diffusion leading to peak broadening. Right: the C-term or resistance to the mass transfer. C_M and C_S are respectively the resistance in the mobile phase and stationary phase.

The resulting curve of the van Deemter equation and the three terms contributing to it are shown in Figure 2.4. From this curve, the optimal mobile phase linear velocity which offers the highest achievable efficiency for a separation can be deducted from the minimum in the curve.

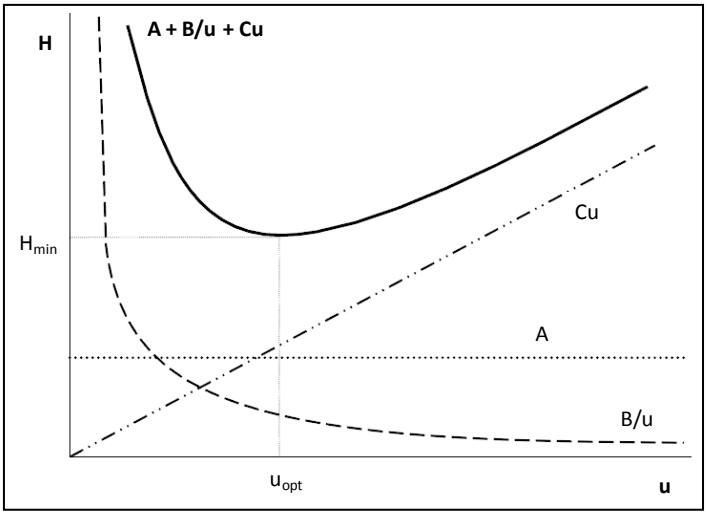

Fig. 2.4 The van Deemter curve with the three contributing terms: A, B/u and Cu. H_{min} is the minimal plate height corresponding to the optimal mobile phase velocity u_{opt}.

Each van Deemter curve is different and specific and depends on the choice of the column and its stationary phase chemistry, the nature of the analyte, the used mobile phase, the column temperature and the particle size of the column packing. Considering the particle size d_p, the shape of the van Deemter curve changes significantly as shown in Figure 2.5. In this work, columns or column segments packed with 5 µm particles are used.

Besides the van Deemter equation, other models which describe peak broadening in liquid chromatography, such as the Knox and Giddings equations, have also been developed [9], [10].

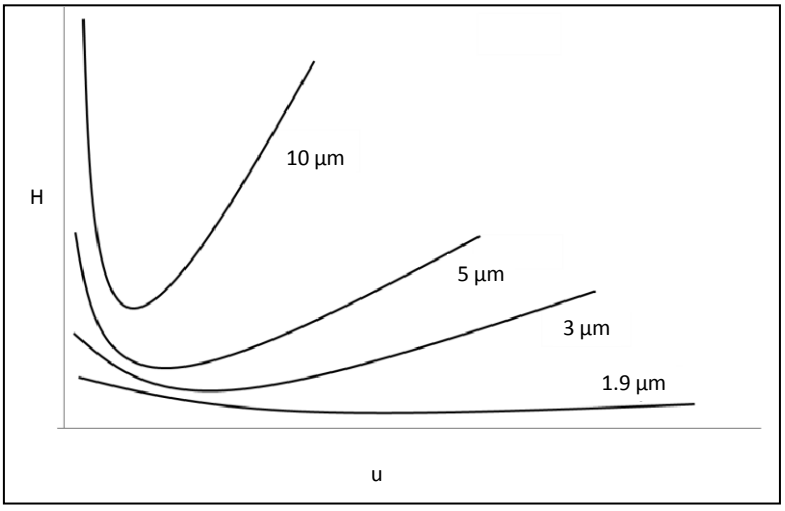

Fig. 2.5 Van Deemter curves for columns packed with different particle sizes.

2.2.4 Resolution

The degree of observed separation between two peaks is best quantified via the resolution R_S. The resolution between two peaks in a chromatogram can be determined through the following equation [4]:

$$R_S = \frac{2(t_{rB} - t_{rA})}{W_{bB} + W_{bA}} \qquad \text{(eq. 2.9)}$$

Equation 2.9 relies on parameters such as retention times and peak widths which can graphically be deducted from the chromatogram.

From equations 2.1, 2.2, 2.3 and 2.9, the "master" equation of chromatography also known as the Purnell equation can be derived [11]:

$$R_S = \frac{\sqrt{N}}{4} \frac{\alpha - 1}{\alpha} \frac{k}{k+1} \qquad \text{(eq. 2.10)}$$

This equation plays a central role in chromatography as it directly represents the influence of the three parameters retention, selectivity and efficiency on the resolution. This is also graphically represented in Figure 2.6. It can be seen in that figure that additional influence of the retention factor on the resolution becomes very limited once it exceeds a value of 4. The influence of efficiency on the resolution is described by a square root relationship between the resolution and the plate number N (equation 2.10). The last decade, a lot of research with the focus on efficiency is performed to enhance the resolution in separations. However, the parameter with the most significant influence on the resolution is the selectivity. There are various ways to tune and optimize the latter, which is elucidated in detail further on in this work.

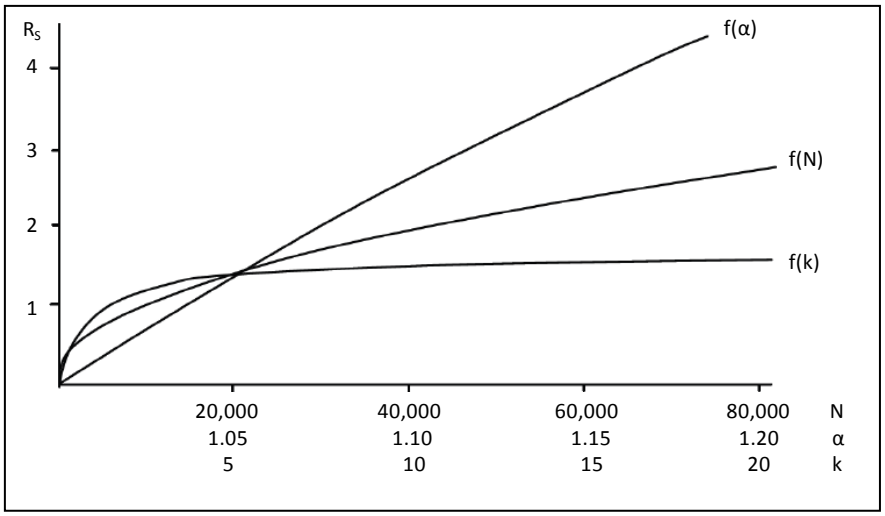

Fig. 2.6 Influence of the retention factor k, the plate number N and the selectivity factor α on the resolution R_S.

2.3 Instrumental aspects of HPLC

HPLC systems minimally contain the following major components: a mobile phase pump, an injector for introduction of a sample, a column and a detector. Besides, some technical parts can be included such as a mobile phase degasser or a column oven. A schematic overview of an HPLC system is shown in Figure 2.7.

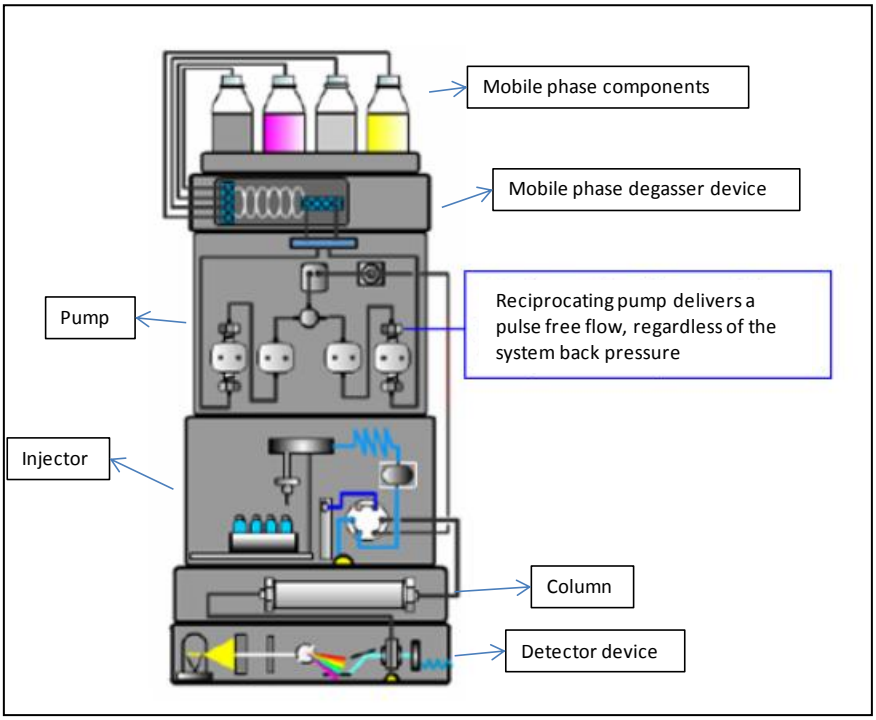

Fig. 2.7 Schematic overview of an HPLC system which contains a storage platform for the mobile phase components, a mobile phase degasser, a pump, an injector, a column and a detector.

For injection, a small amount of sample typically between 0.1 µL and 50 µL (in analytical HPLC) is introduced in the chromatographic circuit or system. The resulting sensitivity or signal-to-noise ratio of a peak apex (c_{max}) can be increased by reducing the column length L, reducing the internal diameter of the column (d_c) and increasing the efficiency N (=L/H) according to following relation (Eq. 2.11) [12]:

$$c_{max} \propto \frac{\sqrt{N} V_i}{L d_c^2 (1+k)} \qquad \text{(eq. 2.11)}$$

Note that an appropriate injection volume V_i is quadratically related to the column diameter. Injection is ideally done without significantly affecting the flow rate and the pressure. In analytical HPLC systems, injection is typically assisted through a 6-way valve system by which the sample is introduced in the mobile phase circuit without disturbance of the flow rate.

The pump in an HPLC system delivers the mobile phase at a certain (constant) flow rate. Nowadays, an HPLC pump should also be able to deliver flows of variable mobile phase compositions, as often gradient elution is applied. A small within and between day variance of the flow rate is important for obtaining repeatable and reproducible retention times and peak areas. In this context, there are two mainstream ways of achieving a gradient profile using HPLC pumping equipment: a binary pump system and a quaternary pump system [13]. In a binary pump HPLC system, two pumps work in unison but each delivers a specific volume fraction of the total flow. The binary pump is often called a high pressure mixing device as the mobile phase components are mixed on the high pressure part of the system. These systems are generally recognized to give the most reproducible gradient profiles, especially at extremes of flow or gradient profile composition. They have the lowest internal mixing volumes and they can mix and deliver gradients very quickly with a very low gradient dwell time. This dwell time is the time needed for the front of a gradient generated by the pump to reach the column inlet and is explained more in detail in Chapter 4. A quaternary pump can deliver up to four mobile phase components simultaneously via a mixing device prior to the pump. The quaternary pump consists of one dual-piston reciprocating pump and a solenoid controlled portioning valve located in-line between the solvent degasser and the pump head. This pump is often called a low pressure mixing pump as the mixing of all mobile phase solvents is done prior to liquid compression. Despite the advantage of the ability of mixing four solvents, the mixing is considered less accurate and reproducible than the high pressure mixing in a binary pump, especially for the extremes of flow or gradient composition as mentioned earlier.

The column is the part of a chromatographic HPLC system where the analytes are separated and is therefore the heart of the chromatographic system. Related to this work and the selectivity offered by columns, a further detailed discussion is presented in section 2.6 and section 2.7.3.

Another essential component of an HPLC system is the detector which should be able to monitor on-line the effluent of a column and generate signals when analytes elute from the column. The resulting plot of the signal versus the analysis time is called a chromatogram. Note that in the early days of HPLC, detection was often performed by collecting effluent fractions and analyzing them off-line by means of gravimetric or wet chemical techniques [14]. From the 1940s and 1950s on, the first on-line detectors for LC such as the refractive index (RI) and conductivity detectors appeared on the scene [15], [16]. From a practical point of view, a detector should be selected fit-for-purpose and thus based on its ability to specifically detect with an adequate level of sensitivity the relevant analytes of a separation application. If the purpose of an application requires the detection of all present compounds, the use of an universal detector or so called bulk property detector is desirable [14]. The search for such a sensitive universal detector for HPLC, much like flame ionization detection

(FID) for gas chromatography (GC), over the years led researchers to adapt GC detectors for use in HPLC [17]–[20]. Too badly, the removal of HPLC mobile phase through evaporation limited any real applicability. The RI detector can be considered as the most common bulk property detector responding to all analytes as it measures properties common to all analytes by measuring differences in the mobile phase with and without the sample. Again too badly, the RI detector is inherently limited in sensitivity as it is the chromatographic equivalent of determining the weight of sailor by weighing the ship before and after the sailor departs for shore leave. While a truly universal HPLC detector with a similar sensitivity as the FID in GC is still elusive, many different types of detectors have been developed. In the 1960s, the first ultraviolet (UV) detector for HPLC was introduced [21]. Subsequent improvements led to better sensitivity and variant upgrades such as variable wavelength and diode array UV detectors [22]. The UV-VIS detector can be considered as an analyte-specific detector. They respond only to analytes with chromophores that absorb UV light at a particular wavelength. With UV detection, the sample concentration, output as absorbance, is determined by the fraction of light transmitted through the detector cell by Beer's law:

$$A = log \frac{I_0}{I} = \varepsilon bc \qquad \text{(eq. 2.12)}$$

where A is the absorbance, I_0 is the incident light intensity, I is the intensity of the transmitted light, ε is the molar extinction coefficient of the sample, b is the pathlength of the cell and c is the molar sample concentration. Examples of other analyte-specific detectors include fluorescence, conductivity and electrochemical [14], [23]. Note that at low UV wavelengths (<210 nm), however, UV detectors can be considered as somewhat universal as about every organic compound absorbs light at such low wavelengths. Other types of detector which lean against universal detection are the light-scattering detector and the charged aerosol detector (CAD) [24], from which improvements in the ability to efficiently nebulize the HPLC column effluent has lead to their increased utility. A popular detection method of light scattering detection is evaporative light scattering detection (ELSD) [23]. A strict requirement with this technique is the usage of volatile mobile phase. The lack of linear calibration curves is a typical observed phenomenon but appropriate calibration and validation allows quantitative analysis over a wide range.

In modern HPLC, universal detection can be aimed at through hyphenation of independent complementary analytical detector techniques to an HPLC system. The most common hyphenation setup is the tandem LC-mass spectrometry (LC-MS) [25], [26]. For a more detailed understanding of LC-MS, the reader is referred to the literature [27]. Other setups such as LC-infrared spectrometry (LC-IR) [28] and LC-nuclear magnetic resonance (LC-NRM) [29] have also been used.

For a detailed overview of the instrumental aspects of HPLC, the reader is referred to one of the many literature handbooks of HPLC [30].

2.4 Separation modes in HPLC

Depending on the nature of the analytes to be separated, an appropriate LC mode can be selected. A short description of various LC modes is given in this section. These LC modes differ from each other according to the used separation mechanism and to the related composition of the stationary phases and mobile phases.

Normal phase liquid chromatography (NP-LC) is the oldest mode of liquid chromatography which was introduced by Tswett [31]. In NP-LC, a polar stationary phase is used such as pure silica, pure alumina, or a silica bonded phase such as cyanopropyl, aminopropyl, diol, ethylpyridine and others. The mobile phase is less polar compared to the stationary phase and is typically a non-polar organic solvent such as n-hexane, iso-octane, ethyl acetate and others. NP-LC is useful for the separation of analytes by the type of polar functional groups which they carry as the separation is based on the adsorption interaction of these polar groups of the analyte with the polar sites present on the stationary phase [32]. The more polar the analyte, the more it is retained on the stationary phase. The separation analysis can be tuned by a wide variety of mobile phase solvents differing in polarity. Increasing the polarity of the mobile phase during the analysis in-situ by means of a gradient program which delivers a varying mobile phase composition consisting of two or more mobile phase components, results in a decreasing retention time of an analyte. As a drawback, this mode is often characterized by a low degree of reproducibility and long equilibration times when using pure silica or alumina as stationary phase [33]. Undesired presence of water can show adsorption onto the adsorption sites on the stationary phase and lead to deactivation of the stationary phase and irreproducible retention times. As a result, NP-LC has lost popularity with the entrance of reversed phase liquid chromatography (RP-LC). However, silica bonded stationary phases mentioned above are less confronted with this irreproducibility issue [34].

Nowadays, reversed phase liquid chromatography (RP-LC) is the most common type of HPLC used [35]. In RP-LC, the stationary phase is hydrophobic. Typically, a silica bonded stationary phase coated with a hydrocarbon chain is used. Besides, polymer or graphitic carbon stationary phases are applied as well, but to a less extend. The mobile phase has a more polar character and consists typically of a mixture of an aqueous component with an organic modifier such as acetonitrile or methanol. Depending on the column used, the separation mechanism is considered to rely majorly on two distinct molecular interactions: adsorption and partition, whereby the former faster mechanism is

favored [36]–[38]. RP-LC is capable of separating a wide variety of analytes, both in molecular weight and polarity. It is used for an extended part (circa 70-80%) of all LC analysis [35], [39]. The addition of counter ions, as used in ion pairing LC, has further expanded the application range of RP-LC. Compared with NP-LC, RP-LC is characterized by a high degree of reproducibility. Column equilibration occurs much faster and analysis with gradients have a higher level of reproducibility.

The last decade, hydrophilic interaction liquid chromatography (HILIC) has gained a lot of interest. It can be considered as an extension of NP-LC where the stationary phase is also polar. The mobile phase, however, consists of typical RP-LC water miscible organic solvents and contains a small amount of water and buffer. The stationary phases are typically pure silica or silica with functional groups such as diol or amide groups anchored to it. The separation is considered to be mainly based on the formation of a pseudo-stationary water layer on the column stationary phase between which fast partitioning occurs with the rich organic solvent mobile phase [40], [41]. For a more detailed elucidation of the retention mechanism and how to tune a separation, the reader is referred to the literature [40]–[42]. HILIC can be applied for the separation of more polar, ionizable or ionic compounds which have a low or no retention on RP-LC columns. An expansion of this mode involves the combination of HILIC with zwitterionic ion chromatography [43], [44]. Retention hereby is mainly offered by the HILIC principle but considerable effects on selectivity can be observed compared to pure HILIC due to an extra assumed ion exchange mechanism.

Ion exchange chromatography (IEC) is used for the separation of ionic and ionizable compounds such as amino acids, peptides, proteins and nucleic acids [45], [46]. The stationary phase is typically equipped with ionic groups. In cation exchange chromatography positively charged molecules are attracted to a negatively charged solid support. Conversely, in anion exchange chromatography, negatively charged molecules are attracted to a positively charged solid support. A distinction in IEC can be made according to the ion type of the stationary phase, whether these are strong or weak acids or bases: strong cation exchange (SCX), weak cation exchange (WCX), strong anion exchange (SAX) and weak anion exchange (WAX). The mobile phase contains counter ions, to push the analytes from the column. Elution is achieved by increasing the ionic strength such as to suppress the ionic interaction. The separation profile can be tuned by varying the pH and the salt gradient during the analysis. One of the main disadvantages of IEC is its buffer requirement whereby a huge amount of salts is needed.

Another LC mode is size exclusion chromatography (SEC) where separation of the molecules occurs based on their hydrodynamic size [47]. The analytes thereby do not interact with the stationary phase, but are separated by their (in)ability to penetrate the pores of the packing. Thus, the pores in

the particles then serve as a filter and smaller molecules will be retained more on the column as they can enter a larger fraction of the pores and spend more time in the station phase. This is a typical LC mode for the separation of polymers and bio-molecules [48], [49].

Another separation mode which can be mentioned in this overview is affinity chromatography. For further elucidation on this mode, there can be referred to the literature [50]–[53].

In this work, the focus has been set on the separation of small organic molecules with RP-LC and more specific how retention can be optimized through the stationary phase, which is an approach beyond the typically applied optimization approaches which are first outlined further in this chapter.

2.5 Influence of the mobile phase compounds and practical considerations

2.5.1 Organic solvent type and strength

As mentioned in section 2.4, the mobile phase in RP-LC typically consists of an aqueous phase and an organic modifier. Sometimes, non-aqueous RP-LC can be performed in the case of very hydrophobic analytes which do not show solubility in aqueous phases and when too high retention is obtained when water is used in the mobile phase [54], [55]. Common water miscible organic modifiers are acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF). Organic modifiers differ from each other in terms of solvent eluotropic strength, which means that they have an effect on the retention of analytes in a different way [56]. THF is a stronger solvent compared to ACN and in its turn, the latter is stronger compared to MeOH. Consequently, a lower percentage of TFH is needed compared to a certain percentage of MeOH or ACN to attain the same k for a given analyte. Besides, eluotropic strength of these solvents also affects the selectivity in a different way. This opens the way for retention and selectivity modeling as will be outlined further in section 2.7.2.1.

From practical point of view, there are also several additional points to be taken in consideration which play a role in the decision of the use of a particular organic modifier or of other mobile phase additives [57]. An essential point of attention is the miscibility of the selected organic modifier with the applied aqueous mobile phase component or other co-solvents or additives. Besides, an organic modifier which shows low solubility for the analyte(s) of interest is preferably not selected as the role of the organic modifier is ideally to control the elution of the analyte and certainly not to precipitate the analyte on the column. Note that for the analysis of large macromolecules such as polymers or bio-molecules such as proteins, avoiding precipitation on the column and obtaining acceptable recoveries is not so effortless [58], [59]. As described earlier in this section, THF is often considered as a third organic modifier of choice, but in practice it is not popular due to its toxicity and its limited shelf-life as it has a tendency to degrade and form peroxides. Further, the use of polyether ether ketone (PEEK) tubing is not recommended with THF as organic modifier as it dissolves the former. The choice of mobile phase components is also related to the detector that is used. With an UV detector, detection preferably occurs at an analyte-specific wavelength for which the analyte of interest shows a sensitive absorbance in the chromatogram. Most solvents are transparent to UV down to a certain wavelength, but below this UV cut-off wavelength they absorb UV light leading to a loss of sensitivity. As a result, this influences the choice of the mobile phase constituents during method development, especially related to constituents such as e.g. THF and formic acid (FA).

Applying HPLC with e.g. tandem MS as detector, the use of THF should also be critically considered before usage. THF is highly flammable and an aprotic solvent which tends to polymerize in ionization sources. In opposite, ACN and MeOH are more user-friendly solvents for MS as they are volatile. Note that MeOH has a protic character which is beneficial for positive electrospray ionization MS.

2.5.2 Isocratic versus gradient analysis

The task of chromatographically separating a complex mixture containing analytes spanning a wide hydrophobicity range with RP-LC can be referred to as "the general elution problem" [60]. Considering the elution mode of a RP-LC separation, a distinction is made between isocratic and gradient analyses. With isocratic elution, the composition of the mobile phase constituents remains constant during the analysis. With gradient analysis, the composition of the mobile phase constituents is varied as a function of time towards a higher fraction of organic modifier in the mobile phase leading to an increase in eluotropic strength. Isocratic elution depicts some advantages such as its simplicity concerning the cost of instrumentation, and the possibility of consecutive injections without the necessity to re-equilibrate the column. However, as its practical usability is limited to the separation of mixture of compounds, encompassing a relatively small polarity range, isocratic elution is often not an appropriate approach. The feasibility of finding conditions, allowing both satisfactory separation and acceptable retention times is low for complex samples. This type of analysis is further complicated by the band broadening and reduced sensitivity of the most retained analytes. In Chapter 3, the applicability of stationary phase optimized selectivity liquid chromatography (SOSLC) in isocratic analysis is assessed.

Gradient elution brings an elegant solution to the general elution problem. As the eluotropic strength is thereby increased as function of time, the elution of most compounds can be much accelerated, resulting in acceptable retention times while often improving the separation. Unwanted peak broadening and decreased signal-to-noise sensitivity is thereby avoided due the to peak compression phenomena involved [61].

The change of the mobile phase composition as a function of time is controlled via a time program. The number of such gradient programs that can be set up, is almost unlimited. Usually, such an increase is linear, but other gradient programs containing several isocratic steps (i.e. multistep-isocratic gradients) or several segments of different slopes (i.e. multi-linear gradients), can be applied as well and contribute to the extremely large number of gradient profiles which can in theory be used.

2.5.3 Buffers and additives

The influence of the addition of buffers or other additives to the mobile is an aspect in HPLC which should not be underestimated. Analytes such as drugs can have either acidic or basic functional groups and can exist in solution in ionized or non-ionized forms. The ionic state and degree of ionization greatly influences their chromatographic retention in RP-LC. Typically, the ionic form does not partition well into the hydrophobic stationary phase and therefore shows significantly lower retention compared to the neutral un-ionized form. From a practical point of view, the first choice for a lot of chromatographers is to work at low pH in RP-LC, in the pH 2-3 range. The rationale hereby is that under these conditions, most acidic samples will have suppressed ionization and be well retained, as will neutral molecules [39]. Common acids used for mobile phase preparations are formic acid (FA), acetic acid (AA) and trifluoroacetic acid (TFA) and adequate dosage leads to these typical pH values between 2 and 3 [57]. Basic analytes are expected to be ionized at low pH and will therefore often result in poor retention unless the bulk of the molecule is non-polar. However, if an analyte has a too polar character and is expected to be unretained in the reversed phase mode, ion pairing chromatography by the addition of an ion pairing reagent can prove to be useful [39], [62]–[64]. The ion pairing reagent has an ionic part and a non-polar tail such as e.g. hexane sulphonic acid. The non-polar part is held strongly by the non-polar stationary phase, leaving the charged functional group open into the mobile phase. An analyte with an opposite charge can be attracted to the immobilized ion pairing reagent, resulting in chromatographic retention [39]. A disadvantage hereby is the sensitive influence of the column temperature and the organic content on the concentration of ion pair reagent in the stationary phase. This means that temperature must be controlled carefully and that gradient elution is very difficult. Also many ion pair reagents have a substantial UV absorbance as explained in section 2.5.1 and it is assumed that they can never be washed entirely from the column leading to the dedication of a column to ion pairing applications. Note that sometimes the ion pairing process is described to take place in the mobile phase, resulting in a neutral ion pair between the analyte molecule and the reagent which is then retained as a neutral species in the standard reversed phase manner [65]. In this context TFA could be called an ion pairing reagents as well, especially with the focus on proteins and biomolecule analysis [65].

Analytes often also have several functional groups resulting in multiple pKa values and often only a narrow pH range is hereby suitable to have the molecule in its neutral form. Therefore, buffers are commonly used to control the pH of the mobile phase for the separation of such acidic or basic analytes. Since a buffer is only effective within ± 1 pH unit from its pKa, judicious selection of the proper buffer within its buffering range is paramount. Note that for the development of MS

compatible HPLC methods, volatile acids and their ammonium salts should be used [27]. Also here, additives such as FA and acetic acid facilitate the generating of retention.

Note that the use of a mobile phase with a high pH is not favorable as a long lifetime of most silica-based columns used in routine analyses or described in pharmacopeial monographs is only guaranteed in the pH range of 2 – 7 [66]. However, the increasing availability of new silica-based columns stable under high and extreme pH conditions (pH 1 – 12) offers an important alternate solution for the separation of basic analytes in R&D stage [67]. This approach gains interest for assay and impurity testing of water soluble basic drugs as the advantages are sometimes improved MS compatibility and better sensitivity compared to the ion-pairing approach.

2.5.4 The influence of flow rate, pressure and temperature

The flow rate of the mobile phase is one of the most important parameters in chromatography. As explained in section 2.2.3 by means of the van Deemter theory, the achievable separation performance is directly related to the linear velocity u ($u = L/t_0$) of the mobile phase through the column. Percolating a flow rate F through a packed column results in a back-pressure ΔP which depends on the factors described in Equation 2.13 [68]:

$$\Delta P \propto \frac{\eta F L}{K_0 \pi d_c^2} \qquad \text{(eq. 2.13)}$$

Hereby, it can seen that ΔP is directly proportional to the viscosity η, the column length L and the flow rate F. An inversely proportional relationship is observed between the specific permeability K_0, the square of the column diameter d_c and the square of the particle size d_p. Because of its low viscosity, a chromatographer will often favor a modifier such as ACN instead of isopropanol. Other considerations also play a role in the selection of the organic modifier such as the eluotropic strength and selectivity as described in sections 2.5.1 and 2.7.2.1. A longer column will require considerably more force to mobilize a liquid through a packed bed. If the column becomes too long, then the pressure might exceed the capability of the pump or the physical stability of the packing and adjustments to the operating conditions must be made. The flow rate is an easy parameter to anticipate on and to adjust whereby a decrease will result in a lowered back pressure. An increase is often proposed to speed up an analysis, but a too high flow rate can again result in overpressuring the column or the pump. And as mentioned before, changing the flow rate can becomes disadvantageous for the separation efficiency. The most significant factors in equation 2.13 influencing the pressure are the particle size d_p and the column diameter d_c. Although the back pressure generated by a column is quadratically related to the column diameter and this equation

creates the illusion that the use of broad columns is preferred, in essence it is demonstrated that analyses performed on columns of any diameter should always generate the same back pressure when these analyses are performed at the same linear velocity. Hence, the optimal flow rate can easily be scaled for each column diameter according to the square of the column radius. By contrast, a reduction in particle size by half leads to a pressure increase by a factor of 4. In practice, the resulting back pressure will hereby often exceed the operating pressure of conventional HPLC systems. Fortunately, when the particle size is decreased, a shorter column length L can also be applied without losing much efficiency (eq. 2.6). On the other hand, as a shift in optimal velocity is observed when the particle size is reduced, more pressure is again needed in order to be able to perform HPLC with smaller particles. To address these issues, an increase of column temperature can also be considered to lower the solvent viscosity η and thus the back pressure and to speed up the mass transfer. With appropriate equipment ultra high pressure LC (UHPLC) conditions can be generated [69]–[71], but frictional heating appears hereby as a considerable phenomenon in a column packed with very fine particles [72]–[74]. Frictional heating is induced by the friction of the mobile phase percolating through the column bed at very high pressure. This heat dissipates along and across the column allowing the formation of axial and radial temperature gradients which may influence the retention and efficiency of the column. Furthermore this influence depends on how the external temperature of the column wall is controlled [75], [76]. From the above discussion, it becomes clear that the choice of the chromatographic conditions and of the used system easily can turn in reciprocal trade-off decisions.

How the selectivity can be affected by temperature and pressure changes, is described in sections 2.7.2.5 and 2.7.2.6 respectively.

2.6 Influence of the stationary phase parameters on the separation

2.6.1 Introduction

As mentioned in sections 3.2.2 and 2.4, HPLC is in the vast majority of cases performed in the packed bed format whereby porous silica particles with sizes ranging from 1.5-5 µm are used. Depending on the used separation mode the silica is or is not derivatized with a number of functional groups to provide the stationary phase with the intended retentive character. In most applications, the silica particles therefore only act as a supportive material for the stationary phase. As a technologic extension, core shell columns gain some interest the last decade. Hereby, the particles consist of a solid core and a porous superficial layer which lead to an increased efficiency without an excessive backpressure. The reduced dept of the outer porous layer limits the diffusional path of analytes, leading to minimized mass transfer resistance and minimized peak broadening. By contrast to packed columns, monoliths are porous rod structures characterized by mesopores and macropores which provide a high permeability, a large number of channels, and a high surface area available for reactivity. The backbone of a monolithic column is composed of either an organic or inorganic substrate, and can easily be chemically altered for specific applications. Monolithic columns depict a different structure and different physico-mechanical properties compared to packed columns [77]. Independent of the separation mode, a number of column characteristics emanate from the supportive material as such which are described here before selectivity tuning aspects are further discussed.

2.6.2 Porosity of the stationary phase

Porous materials are almost always used in HPLC as it allows to dramatically increase the surface area, and therefore the contact area of the stationary phase. This significantly increases the loadability of the sample on the column and the retention of analytes on the stationary phase. Typical silica material depicts surface areas of 200-300 m^2/g which is many orders of magnitude more in comparison to non-porous silica particles of the same size. In that context, the division between micro-porous (pores with diameters < 2 nm) and meso-porous particles (pores with diameters between 2 and 50 nm) can be mentioned. The space between the packing materials, i.e. the interstitial space, is represented by the external porosity ε_e. The space inside the packing material, i.e. the internal space, is represented by the internal porosity ε_i. The combination of these two

porosities is represented by the total porosity ε_t. The total porosity of a column typically lies between 50% and 65% [78].

2.6.3 Permeability

The permeability describes the relationship between the parameters that influence the column back pressure. This is an important parameter because it has a large influence on the operational capability of a column as it refers to a packed column and how easily the mobile phase can be percolated through it. The permeability K_0 of a column can be described by an adapted form of equation 2.13 [68]:

$$K_0 \propto \frac{\eta F L}{\Delta P} \qquad \text{(eq. 2.14)}$$

where η is the viscosity of the mobile phase, F is the flow rate, L is the length of the column and ΔP is the pressure drop over the column. The permeability is inversely proportional to the pressure drop over the column. This indicates that a column that exhibits a high pressure drop at a given linear velocity will display a lower permeability compared to a column exhibiting a lower pressure drop at the same linear velocity.

The permeability of a column can also be calculated using the Kozeny-Carman equation [79]:

$$K_0 = \frac{1}{180} \frac{\varepsilon_e^3}{(1-\varepsilon_e)^2 \varepsilon_t} d_p^2 \qquad \text{(eq. 2.15)}$$

From equation 2.15 it is clear that the permeability of a column is very much related to the particle size and to the external porosity.

Permeability of a type of column, which relates several parameters such as the column back pressure, external porosity, particle size, flow, column length and viscosity, is crucial to allow the possible coupling of multiple columns in series as discussed below [6], [80].

2.6.4 Coupling multiple (identical) columns in series for efficiency enhancement

As mentioned above, the permeability of a column, dependent on the external porosity in the packed bed and on the available pressure that the HPLC system can generate, together with external parameters such as the solvent type, viscosity and temperature determine if HPLC columns of a certain length can be coupled in series or not.

The coupling of columns in HPLC opens the possibility to enhance the overall column efficiency and to tune the selectivity. The former is discussed here and the latter forms a central part in the research performed in this thesis and is discussed further on.

In equation 2.6 [6], it can be seen that an increase in column length L by serially coupling columns can lead to a proportional increase in column efficiency while it will inevitably lead to a concomitantly linear increase in the pressure required to percolate the mobile phase through the columns. Note that coupling columns does not automatically increases the measured plate number as the chromatographist is required to therefore keep working at the minima of the van Deemter curves under all conditions.

The viscosity of the mobile phase can be reduced by increasing the temperature of the chromatographic system (Elevated Temperature LC, ET-LC), but note that his will also affect the shape of the van Deemter curve. This has, for example, been demonstrated by coupling up to eight or even twelve 25 cm x 4.6 mm, 5 µm ODS columns at elevated temperatures on conventional HPLC systems [6], [81]. The maximal number of coupled columns was thereby defined by the maximal pressure drop, which depends on the pressure resistance of the types of column used an on the type of instrumentation. As an example, the reversed phase separation of a polychlorinated biphenyl (PCB) mixture is shown in Fig. 2.8 [6]. The mixture is clearly poorly separated on one column at a flow rate of 1 mL/min (Fig. 2.8.A). On four columns and at a flow rate of 2 mL/min, the analysis time is doubled, as well as the peak capacity (Fig. 2.8.B). Fig. 2.8.C shows the separation on 8 coupled columns in series at a flow rate of 1 mL/min. The increase of the plate number *N* resulted in a significantly better resolution of the peaks. The gradient profile was adapted consequently to obtain the same retention factors.

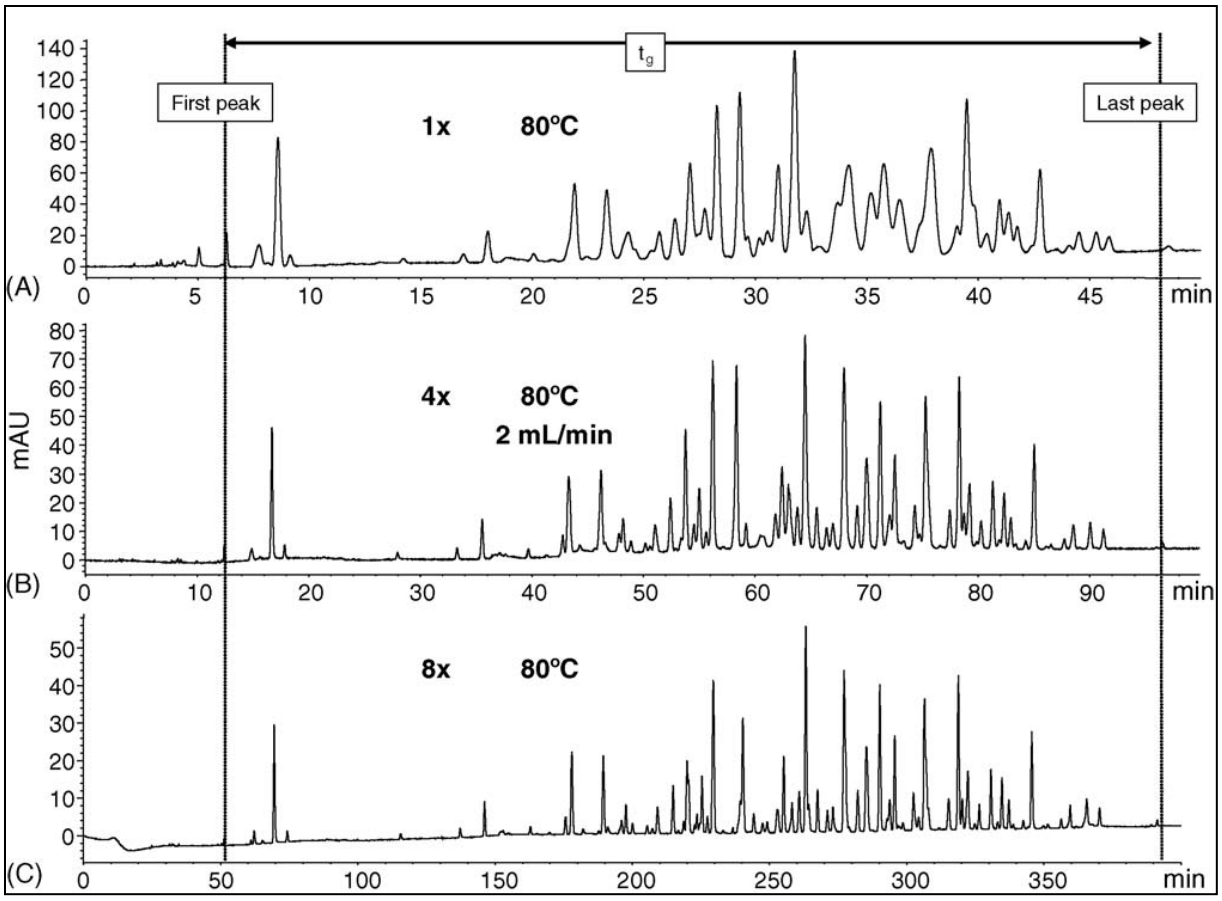

Fig 2.8 Separation of a PCB mixture. (A) one column; (B) four columns at 2 mL/min; (C) eight columns; flow rate: 1 mL/min; temperature: 80 °C [6]

Elevated temperature HPLC is therefore a valuable option for performing high-resolution separations in LC. Longer analysis times can thereby somewhat be compensated by applying increased flow rates which are needed to stay at the minimum of the van Deemter curve. Nevertheless, this approach stays limited to the allowable pressure and temperature limitations of the LC instrument and of the column. It also may often happen that critically unresolved peak pairs are still present in the high efficiency separation due to the intrinsic lack of provided selectivity by the stationary phase of the column and because conventional mobile phase optimization does not offer satisfactory improvements.

In the last decade, a lot of research has been performed on increasing the plate number for enhancing the resolution. As described in section 2.2.4, however, selectivity optimization could and should also play a larger key role in resolution optimization.

Implementing selectivity optimization strategies within e.g. the strategy of coupling columns or column segments containing different types of stationary phases can contribute to a powerful resolution optimization as will be discussed in the following chapters.

2.7 Tools for selectivity tuning in HPLC method development

2.7.1 Introduction

In order to adress a separation problem, multiple decisions concerning non-adjustable factors, such as the choice of the column and of the organic modifier, should be taken. These decisions can be taken, based on intuition, experience, literature or screening. Subsequently, the adjustable factors can be examined and optimized, such as the concentration of the organic modifier for isocratic separations, the pH, the temperature or the gradient program for gradient separations. When fine-tuning of these adjustable factors is not leading to satisfying results, changes are required with regard to the earlier taken decisions which should be reconsidered and the optimization process needs to be performed again. This leads to extra experimental work, unwanted delay in method development and likely more costs. Besides, there is a risk that previously overlapped compounds can be better separated with the new adapted chromatographic parameters, but that other compounds, which were originally sufficiently separated, in their turn start to overlap. This notion suggests that the process to search for satisfactory chromatographic conditions for an optimal separation is not always obvious and that the chromatographist can easily fall into a time consuming trial-and-error approach. To counter the inefficiency of the above approach, several method optimization strategies and guidelines with concern to conventional adjustable parameters have been proposed over the years [82]–[90]. However, in some cases a separation problem can have its own specific bottlenecks and a mixture can be so complex that its separation problem often cannot simply be solved by just following these strategies and guidelines.

Most method development strategies and their optimization approaches are based on empiric results. Generally, this includes two steps. The first step involves modeling. A number of experiments is thereby set up in order to fit equations or train algorithms from the output data which will allow subsequent prediction of retention. The aim is thus to obtain relationships which describe the retention of a compound as a function of the experimental and adjustable factors. This approach leads to a platform or a system that is able to predict and simulate the separation for any arbitrarily chosen chromatographic condition in the valid solution space. The quality of further predictions is set by the number of experiments, by the models and by equations which were used to fit the experimental data. Herewith, the range of experimental factors has an influence on an adequately used type of model and equation, which underlines its contribution to this quality of prediction. In the second step, the predicted or simulated separation is screened for a large number of separation conditions. A condition that is expected to provide sufficient resolution can then be selected. This

output can be facilitated by the use of numerical values given to parameters which evaluates the quality of the separation. The main examples of such evaluation parameters are the resolution and the selectivity. Other examples can be related to the peak shape, such as the symmetry or the tailing.

The more factors that are considered for optimization, the more experimental effort is needed in order to model and to achieve accurate predictions [91], [92]. Therefore, the number of optimized factors should be as reduced as much as possible. It can be that the optimization of one factor (e.g. the organic modifier concentration in isocratic elution) is sufficient to achieve a satisfactory separation [93]. Gradient elution in his turn implies the quest of a suitable gradient program, which is usually more complex in terms of mathematics.

Several software packages are commercially available to facilitate and accelerate RP-LC method development. These packages mostly comply with the existing optimization strategies, guidelines and mathematics. Two examples are DRYLAB [94] and CHROMSWORD [95]. As mentioned, such software packages are aiding tools and are not a watertight guarantee for success.

In what follows, the role of the mobile phase composition and later on of the stationary phase components on a separation is described. Then an overview of adjustable factors to optimize the selectivity is provided. Herewith, special attention is devoted to the prediction of retention, which is the factor that most deeply affects the quality of a chromatogram.

Subsequently, the role of the column and its stationary phase, which is usually considered as an non-adjustable factor, is scrutinized.

2.7.2 Rationalization of the role of the mobile phase in HPLC

2.7.2.1 Selection of the organic modifier composition in the mobile phase

In HPLC and particularly in RP-LC, the choice of the mobile phase components has a strong influence on the retention and selectivity of compounds. This choice involves an aqueous component and an organic modifier component. The ratio of this composition is constant in isocratic analysis and variable as a function of time in the case of gradient analyses.

The pioneering work about the selection of the most appropriate solvents to obtain an optimal mobile phase composition or gradient profile was initiated in the 60's by Rohrschneider who made an extensive study of the properties of various solvents [96]. Inspired by this work, a well-known classification model of organic modifiers was proposed by Snyder [97]. Herewith, the polarity index P'

was introduced which can be defined as a measure for the possibility of a solvent to interact with several polar test compounds:

$$P' = log(K_g'')_{ethanol} + log(K_g'')_{dioxane} + log(K_g'')_{nitromethane} \quad \text{(eq. 2.16)}$$

K''_g are thereby the corresponding solubility constants. Additionally, extra selectivity parameters x_e, x_d and x_n are introduced and can be defined as:

$$x_e = log(K_g'')_{ethanol} / P' \quad \text{(eq. 2.17)}$$

$$x_d = log(K_g'')_{dioxane} / P' \quad \text{(eq. 2.18)}$$

$$x_n = log(K_g'')_{nitromethan} / P' \quad \text{(eq. 2.19)}$$

These values x_e, x_d and x_n represent the relative possibility of a solvent to interact as respectively a proton-donor, a proton-acceptor or as a dipole. These values are usable to select organic modifiers which have different chemical interactions with compounds. Modifiers with the largest possible differences in interactions should have a different influence on the selectivity of a separation. In this way, the selectivity of a pair of compounds can be tuned by varying the organic modifier. This classification of organic modifiers is visualized through the Snyder triangle (Figure 2.9).

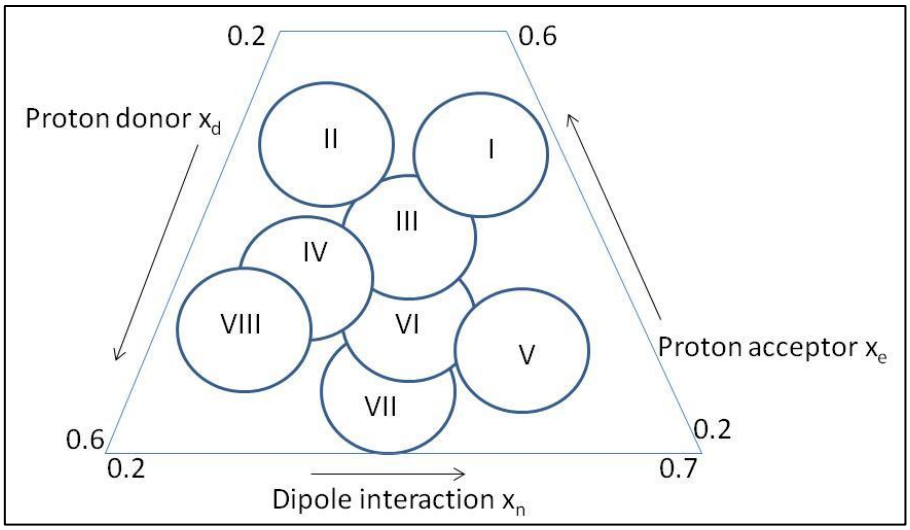

Fig. 2.9 The selectivity triangle of Snyder for organic modifiers[97]**.**

In principle, three solvents, one with 100% acidity, one with 100% basicity and one one with 100% dipolarity, would be located at the extreme corners of the triangle. If these solvents existed, chromatographists should be able to blend them in different proportions and get the properties of solvents lying within the boundaries of the triangle. Unfortunately, such solvents do not exist. As most solvents depict a mixture of acidic, basic and dipolar properties, they are positioned away from

the corners and usually away from the edges of the triangle. As mentioned, often used organic modifiers are methanol, acetonitrile and tetrahydrofuran. These three solvents were already popular solvents before Snyder's work was completed, but since the triangle it is also understood why they are suitable. Methanol is situated in group II which represents alcohols, acetonitrile is situated in group VI which represents the nitriles and tetrahydrofuran is situated in group III together with amides, pyridine and other related structures. Group I represents ethers. Group IV represents acetic acid, formamide, benzyl alcohol and ethylglycol. Group V represents chlorinated solvents. Group VII represents aromatics and aromatic ethers and Group VIII represents water and solvents such as chloroform and tri-ethylene glycol. In theory, solvents situated in more extreme locations could have been expected to be more popular but their use seemed to be practically limited for use in HPLC due to, for example, a lack of miscibility with each other.

As an illustration, the effect of changing the organic modifier on the retention of a number of test compounds is described in Table 2.1 [56]. The applied compounds are benzene derivates. The column used in the example contained a C_8 stationary phase.

Table 2.1 Illustrated influence of the organic modifier [56]

Mobile phase I : MeOH – H_2O (50:50); pH: 4.3; temperature: 30 °C
Mobile phase II : ACN – H_2O (30:70); pH: 3.5; temperature: 30 °C
Mobile phase III : THF – H_2O (25:75); pH: 3.6; temperature: 30 °C

Component (Benzene – R)	Mobile phase I k	Mobile phase II k	Mobile phase III k
-H	3.19	6.78	8.28
$-CH_3$	6.38	13.62	16.12
$-CH_2CH_3$	12.35	27.20	30.20
$-CONH_2$	0.35	0.46	0.50
$-CH_2OH$	0.96	1.06	1.43
-OH	0.96	1.59	3.70
-CHO	1.28	2.77	2.53
-CN	1.47	3.62	3.48
$-CH_2CH_2OH$	1.47	1.58	2.10
$-COCH_3$	1.60	3.11	2.58
$-NO_2$	2.04	5.42	6.61
$-OCH_3$	3.00	6.67	7.26
$-CO_2CH_3$	3.29	6.19	5.32
-Cl	6.54	14.44	18.64
$-CO_2CH_2CH_3$	6.48	12.47	10.15
$-CO_2CH(CH_3)_2$	11.93	24.67	18.31

Parallel with the influence of the organic modifier, a comparison can be made of different mobile phases, but with similar eluotropic power. Herewith, weighing factors for the solvent strength are applied [56]. In Table 2.2., the compositions of the mobile phases H_2O-ACN and H_2O-THF are described in such a way that they are iso-eluotropic with 100% methanol. Note that these weighing factors are only applicable in terms of general retention of the investigated analytes. Individual selectivity changes or exceptions remain of course the power of varying the organic modifier.

Table 2.2 Solvent strength weighing factors [56]

Solvent	Strenght in RP-LC
Water	0
MeOH	2,6
ACN	3,2
THF	4,5

Solvent strenght of 100% MeOH = 2,6
Solvent strength of H_2O – ACN = (1 – x) . 3,2 = 2,6 x = 0,18
Solvent strength of H_2O – THF = (1 – x) . 4,5 = 2,6 x = 0,42

Subsequently, H_2O – ACN (18:82) and H_2O – THF (42:58) are iso-eluotropic with 100% MeOH.

Another similar but three-dimensional approach was developed by Nyiredy with the PRISMA model for determining the optimum solvent strength S_T together with the selectivity of the mobile phase for isocratic separations (Figure 2.10) [98]–[101].

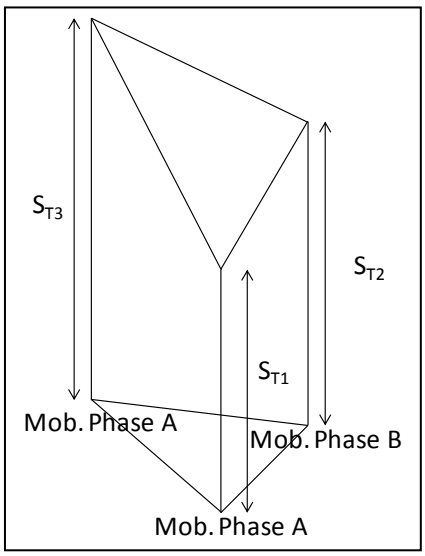

Fig. 2.10 PRISMA model of Nyiredy for optimization of the solvent strength S_T together with the selectivity.

The height of each edge of the prism thereby corresponds to the solvent strength of each solvent. The resulting top triangle will differ in shape from the base triangle because the solvents have unequal solvent strengths. This top triangle is then considered as the solution space in which the selectivity has to be optimized.

The principle of the described triangle and prism for mobile phase optimization is extrapolated to stationary phase optimization as will be described in section 2.7.3.4 and further on in this work.

2.7.2.2 The relationship between the organic modifier fraction in the mobile phase and the analyte retention

In RP-LC, the organic modifier fraction in the mobile phase is a major factor to be optimized, as it influences both the retention and selectivity in a drastic way. Hence, obtaining reliable retention models related to this factor is essential. Several models for describing the retention of analytes for a variable fraction of the organic modifier have been proposed [98]–[109]. Although there is a lack of a truly fundamental description of the relationship between the retention of an analyte and the fraction of organic modifier in the mobile phase, it has empirically been shown that the retention can best be modeled accurately by means of a quadratic relationship between the logarithm of the retention factor k and the volume fraction φ of organic modifier in the mobile phase:

$$ln(k) = a\varphi^2 + b\varphi + c \qquad \text{(eq. 2.20)}$$

where a, b and c are regression coefficients with characteristic values for a given compound on a defined chromatographic system with a particular column and mobile phase components [107].

Within a small range of the organic modifier fractions, this equation can be reduced to a linear equation which leans to the linear solvent strength (LSS) model of Snyder [110]:

$$ln(k) = b\varphi + c = ln(k_w) - S\varphi \qquad \text{(eq. 2.21)}$$

b and c are hereby regression coefficients, analogously defined as in eq. 2.20. With relation to the LSS model, the intercept $ln(k_W)$ can thereby be explained as an estimation of $ln(k)$ at 100% aqueous mobile phase. The slope S is a measure for the sensitivity of retention, which changes as a function of the organic modifier concentration which is directly related to the eluotropic strength. S is a constant for a given compound. Deviations from this linearity are becoming significant at the higher and at the lower organic modifier concentrations. Generally, the benefits of both equations, eq. 2.20 and eq. 2.21, are still matter of debate among chromatographists. On the one hand, a simple equation for retention time prediction algorithms is preferred. On the other hand, an equation that is valid for a

wide range which can lead to an improved bias on the prediction is preferred. However, to counter such subjectivity, statistical tests could be used for the evaluation of fitted models [111]–[113], but the fit-for-purpose depending on the intended application should be the main criterion for evaluation of the model.

Retention in ternary systems, composed of an aqueous component and two organic modifiers, can be described by similar equations [114]–[116]:

$$ln(k) = a_{11}\varphi_1^2 + a_{22}\varphi_2^2 + a_{12}\varphi_1\varphi_2 + b_1\varphi_1 + b_2\varphi_2 + c \quad \text{(eq. 2.22)}$$

where φ_1 and φ_2 are the concentrations of the two organic modifiers in the ternary mobile phase.

As an illustration, the high reliability of retention time prediction under isocratic conditions making use of the quadratic model is demonstrated in Figure 2.11. Note that from practical point of view, an initial scouting gradient run over the whole applicable range is helpful to select adequate and workable isocratic levels for optimal operation [117].

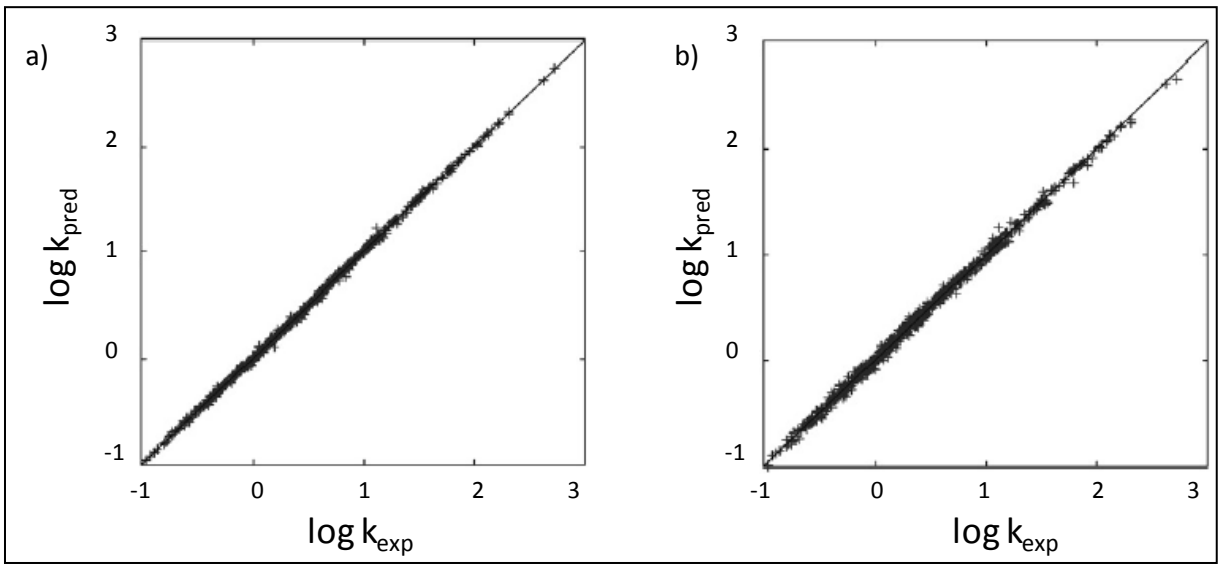

Fig. 2.11 Performance of: a) Eq. 2.20 (r^2 = 0.9994) and b) Eq. 2.21 (r^2 = 0.9974) in the prediction of 752 retention data, corresponding to 152 compounds eluted with methanol-water mobile phases. [117]

Other less known linear relationships have also been described by relating for example ln(k) with polarity parameters that depend on φ, such as the Dimroth-Reichardt parameter E_T [118]–[122].

2.7.2.3 Retention of an analyte under gradient elution conditions

During gradient analysis, the composition of the mobile phase constituents is varied as a function of time towards a higher portion of organic modifier and thus the eluotropic strength is increased. Regarding selectivity, the gradient program is a factor which can be optimized in gradient RP-LC mode. It has a large impact on both retention and selectivity, in compliance with the organic modifier concentration in isocratic RP-LC mode.

The retention time of a compound for a given gradient program in RP-LC can be described through the following equation for gradient elution [107]:

$$t = \int_0^{t_g - t_0} \frac{dt}{k(\varphi(t))} \qquad \text{(eq. 2.23)}$$

where t_0 is the dead time, t_g the retention time under gradient conditions and k(φ(t)) the equation that describes the compound retention as a function of time. This equation postulates that for any gradient, the retention time can be calculated if the function k(φ(t)) is known. This concept incorporates two equations: the variation of the concentration of the organic modifier with the time (i.e. the gradient program), and the dependency of k on φ (i.e. the retention model). Therefore, eqs. 2.20 and 2.21 can be applied to gradients.

If the gradient only comprises a variation in φ described by a linear gradient program, and the relation between ln(k) and time is also linear, then eq. 2.23 leads to the following algebraic solution [123]:

$$t_g = \frac{t_0}{b}\log(2.3k_0 b + 1) + t_0 + t_D \qquad \text{(eq. 2.24)}$$

where b is a parameter related to the solvent strength (i.e. the slope of the gradient program, φ') and t_0 through:

$$b = S\varphi' t_0 \qquad \text{(eq. 2.25)}$$

with

$$\varphi' = \frac{\Delta\varphi}{t_G} \qquad \text{(eq. 2.26)}$$

where Δφ represents the difference between the starting and final organic modifier concentrations. t_G is the gradient time, which is the time when the gradient program reaches the final organic modifier composition. In eq. 2.24, t_D represents the dwell time, which is the time required for the gradient front to reach the column inlet. This means that the dwell time is an automatically present

small isocratic stage at the start of any gradient profile. k_0 corresponds the retention factor of the compound at the organic modifier concentration at the start of the gradient.

In the case of a quadratic retention relation, eq. 2.23 lacks an algebraic solution. To obtain a gradient retention time t_g, numerical integration can be applied [124], [125]. A numerical integration approach is elucidated in detail in Chapter 4.

Another empirical retention relation, which goes beyond a limited linear relation but can lead to an algebraic solution in Eq. 2.23, has been proposed by Neue in 2010 [126]:

$$ln(k) = ln(k_{00}) + 2\,ln(1 + a\varphi) - \frac{B\varphi}{1+a\varphi} \qquad \text{(eq. 2.27)}$$

k_{00} thereby represents the extrapolated intercept, *a* is a curvature coefficient and B is the slope of this relationship. This model allows to fit a curved relationship between the logarithm of the retention factor and the solvent composition. Note that if the curvature coefficient *a* becomes equal to 0, the relationship becomes a classical linear relationship.

2.7.2.4 The modeling of pH as an experimental factor which has an influence on retention

For many compounds, the pH can be an experimental factor with a large influence on retention and selectivity. When a sample mixture contains compounds with weak acidic or weak basic functional groups, drastic changes in selectivity are obtained when changing the pH. However, retention and selectivity as a function of the pH is not easy to model [127]–[148].

In one of the approaches, RP-LC retention of an ionizable compound is described through the weighted mean of the composing basic and acidic species:

$$k = k_A \frac{1}{1+K_h} + k_{HA} \frac{K_h}{1+K_h} = \frac{k_A + k_{HA}K_h}{1+K_h} \qquad \text{(eq. 2.28)}$$

where k_A and k_{HA} are the retention factors of the protonated and of the non-protonated species respectively. K_h represents the protonation constant. As each species is characterized by a different retention, a pH value in the mobile phase medium close to log K, will induce a significant change in retention. This is visualized in Figure 2.12 with aniline (pKa of the conjucated acid = 4.2) as example. Depending on the induced charge by pH-tuning, an increased, decreased or constant retention can then occur. This process of protonation covers multiple pH units and is situated around the log K.

Peak crossing by the retention drops or increases of one or more compounds, while the retention of other compounds remains constant is the strong benefit of the pH as experimental factor in selectivity optimization.

Besides, some inevitable issues should be considered. When the mixture to be separated is complex and the pH is considered as a factor, the chromatographist can encounter a low level of robustness towards selectivity, due to the hardness of adequate modeling. Some variable pH experiment data has been reported, but the approach has been rarely used in practice in RP-LC [135].

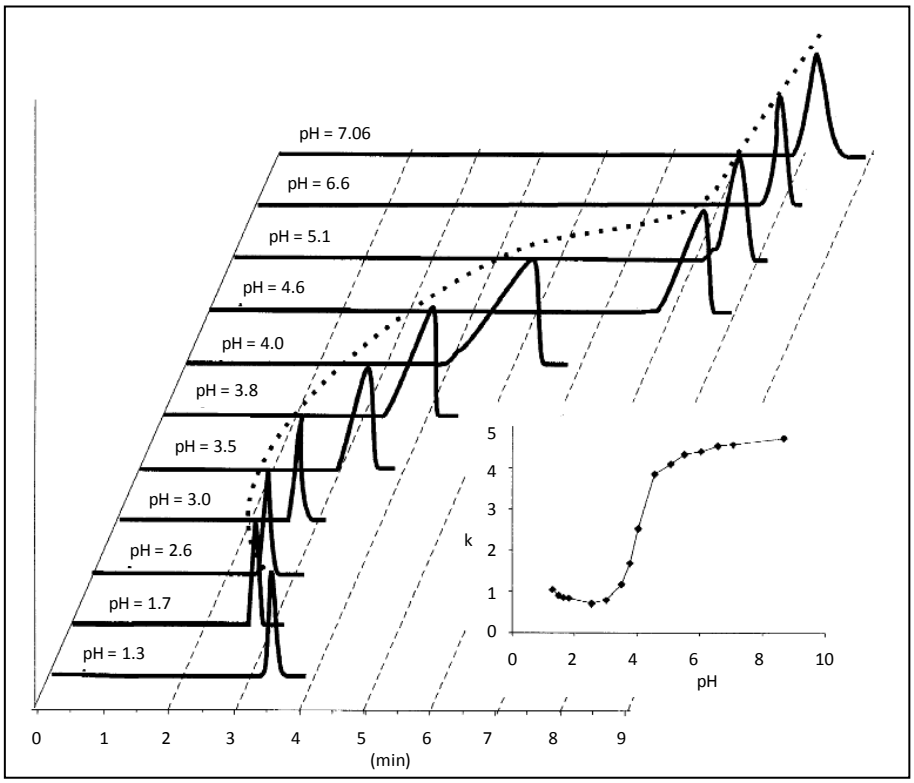

Fig. 2.12 Effect of pH on retention of aniline on a (150 x 4.6 mm, 5 µm) Zorbax XDB-C18 column (mobile phase: acetonitrile – 10 mM disodium hydrogenphosphate buffer adjusted with perchloric acid, pH 1.3-7.1 (10:90), flow rate: 1.0 ml/min, 25°C, UV 254 nm, 1 µl injection)[246]**.**

A practical limitation of this type of strategies is that the working pH range of a lot of columns and stationary phase is limited (e.g. pH 2-7 for conventional RP-LC columns). Also, different compounds can require different pH regions for good separation. As a result, the complexity of searching applicable pH regions increases with the number of pH sensitive compounds in the mixture. By experience, the phenomenon of the presence of two peaks in a chromatogram because of the presence of the two species of one compound when working at a pH close at the pKa value of this compound, can occur and is considered unwanted as it can lead to wrong results and application interpretations. At last, the peak shape of protonated or deprotonated compounds can have large unwanted proportions of peak tailing.

2.7.2.5 Temperature as an optimizable factor

Temperature is a parameter which has well been studied in LC [149]–[153] and it is clear that it has an influence on the retention of an analyte [154]–[156]. It is well known that the viscosity of an HPLC mobile phase is decreasing as a function of increasing temperature and that the diffusion of a compound can be accelerated by increasing the temperature. This can lead to reduced retention times, which may influence and degrade the selectivity. However, relative enhancements in peak efficiency leading to increased resolution have also been reported [154]. The latter is, however, very much connected to the shape of the van Deemter curve at the various temperatures. The role of temperature with relation to retention can be described by the Van't Hoff equation:

$$\log k = \frac{\Delta S}{2.3R} - \frac{\Delta H}{2.3RT} + log\phi \qquad \text{(eq. 2.29)}$$

where ΔS and ΔH represent the entropy and enthalpy variations of the system. T is the absolute temperature, R is the universal gas constant, and ϕ is the phase ratio of the column. The Van't Hoff equation can be rewritten in a chromatographically more convenient way as:

$$\log k = c_0 + \frac{c_1}{T} \qquad \text{(eq. 2.30)}$$

This equation is valid for a workable range up to 90 °C in RP-LC [157]. For wider ranges, the relation becomes more curvilinear, which leads to the implementation of a third term [156]:

$$\log k = c_0 + \frac{c_1}{T} + \frac{c_2}{T^2} \qquad \text{(eq. 2.31)}$$

The practical benefits of temperature as a selectivity tuning factor are currently still debated [157]. If the slope of the Van't Hoff equation is sufficiently divers for different compounds, then changes in temperature allow to influence the selectivity. Herewith, the reversal of the elution order of two peaks is not uncommon. Selectivity changes by temperature are larger for ionizable and polar compounds [158] and intense changes in selectivity are reported for large molecules, such as proteins, as these can turn into different conformations, depending on the temperature [159]. Temperature and eluotropic strength can be considered as orthogonal effects for selectivity optimization [157]. A peak pair which cannot be separated by means of optimizing the eluotropic strength or mobile phase, can sometimes be successfully separated through temperature optimization [142], [159]–[163]. Although it is considered that temperature does not generate a same level of influence on selectivity as the organic modifier concentration, the gradient program, the solvent type or the pH, it is a valuable complementary optimization factor, once the possibilities offered by the previous mentioned factors have been exhausted.

2.7.2.6 The influence of pressure on retention and selectivity

The relation between flow rate, pressure and temperature is outlined in section 2.5.4. Often, chromatographists consider the observed pressure in HPLC separations as an artifact of the separation conditions. If the flow rate is doubled, a doubled back pressure is expected. Or doubling the column length will double the back pressure. Concerning the particle size, the pressure is inversely related to the square of this parameter. Nowadays, a method transfer between Ultra High Pressure (UHPLC) and HPLC is an often occurring phenomenon as the involving laboratories might have their own HPLC equipment with specific features. With this transfer, large changes in pressure are observed as the flow rate, the column length and the particle size are varied. Observing resulting chromatograms of an HPLC separation and its "equivalent" UHPLC separation, some peaks can move relative to each other [164]. This phenomenon, called pressure selectivity, was already observed in the early days of HPLC [10], [165]. Thereby, it is pointed out that pressure changes can affect the molar volume of solutes and thus their interaction with the stationary phase. Frictional heating, mentioned in section 2.5.4, can thereby also participate to observed changes in selectivity when varying the pressure [166], [167].

Modeling retention as a function of the (induced) pressure is not obvious, although the current presented studies claim some observations and conclusions [166]–[177]. In RP-LC, the retention increases for a lot of molecules when the pressure is increased. These retention increases affect polar and ionized compounds more than non-polar compounds of the same molecular weight. The retention of larger molecules was also more affected than smaller ones of the same charge. Concerning the stationary phase, this retention effect is more dramatic for C18 columns than for shorter-chain columns. For HILIC, the observed effects are the opposite of RP-LC and retention is reduced with increased pressure. With the today's current lacking understanding and modeling of pressure selectivity, it is still not a recommended tool to control separations in a practical way. However, pressure-retention effects should not be ignored, especially when methods are transferred and used in both a UHPLC and conventional HPLC environment.

2.7.3 Exploitation of the stationary phase choice for selectivity tuning

2.7.3.1 Introduction

Although the discussion in section 2.4 leaves the reader with the impression that HPLC method development requires only the selection of the correct separation mode followed by optimization of the adjustable parameters, it cannot be sufficiently stressed that for a given type of stationary phase,

a vast number of varying selectivities can be observed in HPLC. Only for the category of C18 columns, today over thousand phases are commercially available [178], [179]. A major ensuing problem of this fact is that it proves extremely challenging to transfer validated methods to other e.g. more recently developed types of stationary phases.

2.7.3.2 Column selection

The large amount of columns makes an adequate decision in terms of column choice not always trivial. On a first view and spread over a range of different stationary phase chemistries, there are a lot of similar columns for each stationary phase chemistry in this giant collection. However, experience learns that even columns with a same stationary phase chemistry but from a different manufacturer can give rise to different selectivities [180]–[182].

Several attempts have therefore been made to produce test conditions to characterize this huge number of stationary phases available [183]–[198]. However, to date, a unified column characterization approach has not been agreed upon by chromatographists or the column manufacturers. An early attempt to do so was introduced by Tanaka et al. [199]. Since then, the USP Working Group on HPLC Columns, the Impurities Working Group of the PQRI Drug Substance Technical Committee in collaboration with Snyder [200]–[208], the NIST Standard Reference Material (SRM) 870 [209]–[211] and the group of Euerby and Petersson [212]–[218] have expanded this type of work. These groups have all attempted to create a testing protocol and a rationale that can assess the most important chromatographic properties of a stationary phase and which can in this way facilitate method translation.

Most of these approaches have utilized various chemometric and statistical approaches to visualize the similarities and dissimilarities of the stationary phases based on Principal Component Analysis (PCA) or by computing a numerical similarity factor as a measure of a stationary phase's equivalent or complementary chromatographic selectivity. Developed column characterization databases can be used in this way to rapidly indentify similar or dissimilar stationary phases. An example of such a database is based on the hydrophobic subtraction model (HSM) and is available *on-line* [168], [169]. A set of five parameters is thereby used to characterize the nowadays commercially available RP-LC columns. These five parameters (H, S*, A, B and C) can be summarized in an equation resulting in an overall covering parameter F_S which can be used for comparing the similarity of two columns [169]. As an application, stationary phases with orthogonal selectivity can then be selected for method development, involving selectivity tuning and optimization. As another application and depending of an analysts objective, a series of similar columns can be selected for which importance is set on one

of the five parameters by means of a weighing coefficient. For example, it can be that an analyst needs a column which only offers a high level of hydrophobicity. By filtering a selection of columns which fulfill this requirement, the analyst can then check in a way if he has one of the adequate columns available in the laboratory.

- H parameter is a measure of the phase hydrophobicity
- S* is a measure of the resistance of the stationary phase to penetration by a solute molecule
- A is a measure of the hydrogen-bond acidity of the phase
- B is a measure of the hydrogen-bond basicity of the phase
- C is a measure of the interaction of the phase with ionized solute molecules

However, the detrimental situation created by the availability of such a vast majority of stationary phases, and the implications it has on the transfer of methods, can also be turned into one's benefit if different selectivities are required.

Considering the range of different stationary phase chemistries, some effects are shortly mentioned. A change in the chain length of an R-group does not drastically influence the retention and selectivity. Larger effects with relation to retention and selectivity are observed when for example a stationary phase with a C_{18} chain is compared with a C_{18} phase with embedded polar groups such as amide groups. Separation on a pure C_{18} or octadecyl silica (ODS) stationary phase is mainly based on hydrophobic interaction. Stationary phases with embedded polar groups have a more preferred retention for proton donors such as phenols or sulfonamides. Phenyl stationary phases for example have an increased retention for π-acceptors because of the π-π interactions. With a cyanopropyl stationary phase for example, the hydrophobic interaction is very low, but on the other hand a good polar selectivity is offered. In the case of C_{30} stationary phases, selectivity can be offered to separate planar analytes from non planar analytes based on shape selectivity.

2.7.3.3 Selectivity tuning in a system by combining stationary phases

The discussion in the previous section illustrates that the much differing selectivities between stationary phases can be exploited in a satisfactory way. An elegant application of these phenomena is the combination of stationary phases in packed columns on individual particles [219], in the column as a mixed bed [219] or by coupling columns containing different stationary phases. Although the first two approaches involve the combination of stationary phases on one particle or in one column is elegant, these two approaches offer only a limited flexibility.

Concerning the third approach, the concept of serially coupling 2 columns with different retention characteristics already goes back a few decades but can be very useful. By contrast serially connecting columns containing different stationary phases can offer an improved selectivity for a critical peak pair that remains unresolved on a single column. In order to add an extra parameter to be optimized for selectivity tuning, a set-up with a T-connector between 2 serially coupled columns is described several times (Fig. 2.13) [220]–[225]. Retention and selectivity can be tuned by varying the individual mobile phase flows in the coupled columns which are or can be packed with different stationary phases.

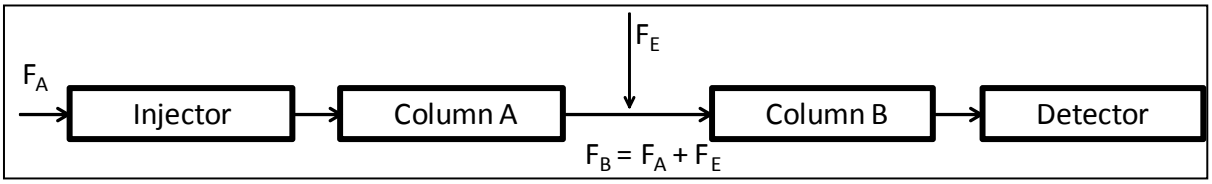

Fig. 2.13 Set-up of the mobile phase flows in the dual HPLC column series. F_A: flow of the mobile phase in column A; F_E: flow of the mobile phase into the T-connector; F_B: flow of the mobile phase in column B.

An interesting upgrade of this set-up with hyphenation of RP-LC together with HILIC for broadening the elution window was investigated and developed by Louw et al (Fig. 2.14) [226].

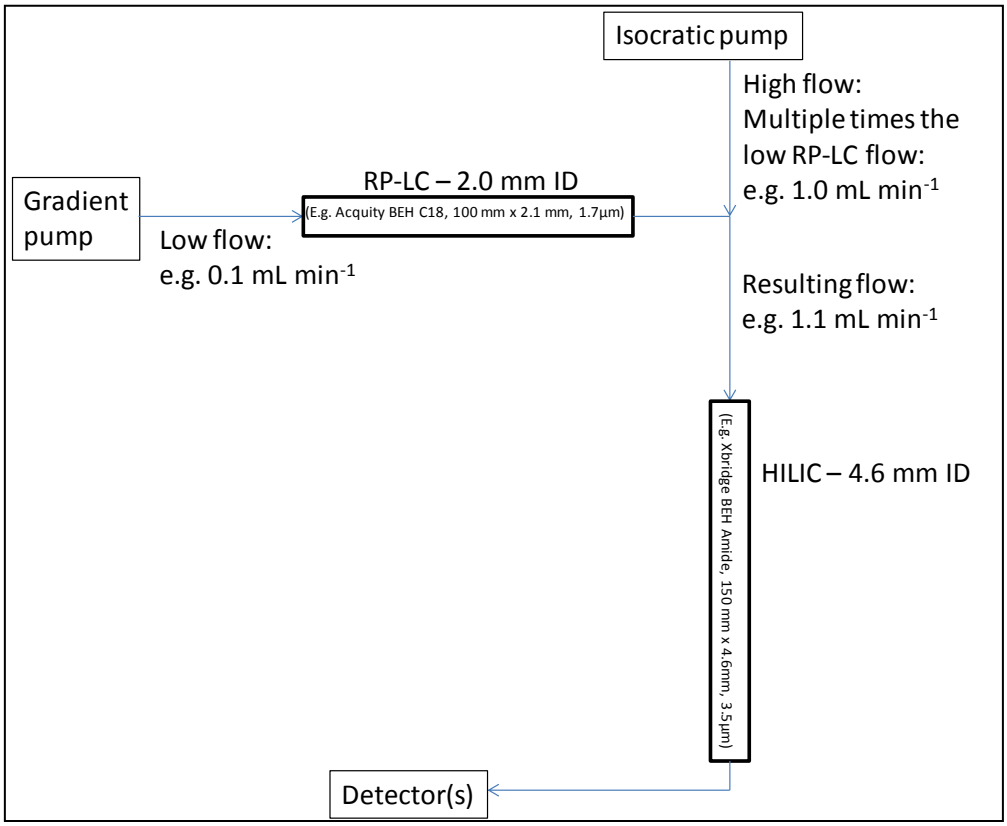

Fig. 2.14 Set-up of a dual HPLC column series, combining RP-LC with HILIC [226].

HILIC experienced much additional interest the past few years, as it is considered as orthogonal to and complementary with RP-LC. Aqueous RP-LC is considered a suitable technique for compounds exhibiting log(octanol/water) or log(P) coefficients between 2 and 8. As aqueous normal phase or HILIC allows retention and separation of compounds with log(P) values smaller than 2, both techniques are considered orthogonal and complementary, especially for pharmaceutical analysis. For a practical attainability, the first column in this set-up is a 2 mm I.D. RP-LC column, depicting an optimal flow rate between 0.1 and 0.2 mL/min. Via the T-piece, the flow for the second column, which is the HILIC column with an I.D. of 4.6 mm, is adjusted to 1.5 – 2.0 mL/min with an acetonitrile rich mobile phase. Therefore, even if the RP-LC analysis part is operated in gradient mode, starting with a mobile phase with high water content, the HILIC column is always operated at high acetonitrile concentration which is required to obtain retention in the HILIC analysis part. The separation of a test mixture, containing both carbohydrates and sulfonamides, is shown in Figure 2.15 [226]. Compared to the single RP-LC analysis, the sugars are separated on the RP-LC-HILIC analysis, while the separation of the sulfonamides remain unaffected.

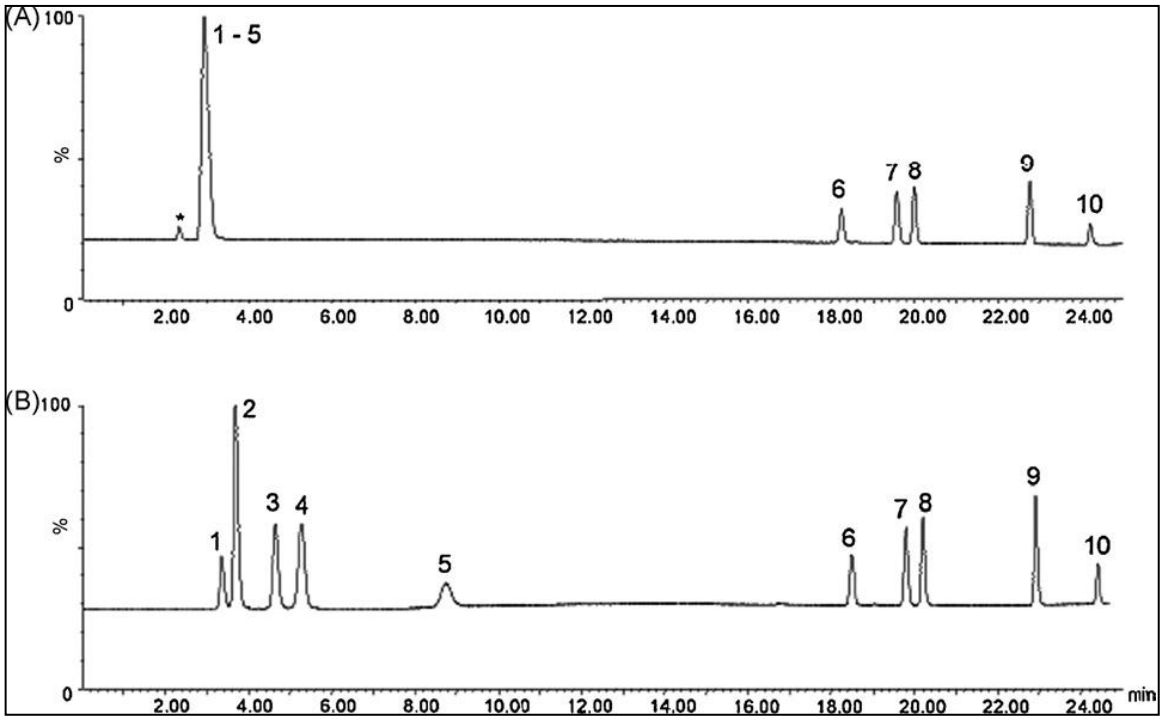

Fig. 2.15 Separation of sugars and sulfonamides. (A) RP-LC gradient analysis; (B) gradient RP-LC/isocratic HILIC analysis. Compounds: (1) ribose, (2) glucose, (3) sucrose, (4) lactose, (5) raffinose, (6) sulfamerazine, (7) sulfamethizole, (8) sulfamethazine, (9) sulfamethoxazole and (10) sulfaquinoxaline [226]**.**

Although very useful, all of the above approaches remain experimental and essentially based on a trial and error approach. Therefore there is need for a rationalization of the column coupling approach as discussed in the next section.

2.7.3.4 Stationary phase optimized selectivity Liquid Chromatography by means of serially coupled column segments (SOSLC): Isocratic elution mode based on the PRISMA model

As mentioned before, the choice of column with a specific stationary phase is the first step in the development of a chromatographic separation and is in the conventional paradigm considered as an invariable parameter in LC. The mobile phase is thereby considered as a variable parameter to be optimized as it can continuously be varied through intermediate mobile phases to adjust the separation.

In order to be able to consider the stationary phase as a variable parameter as well, there should be a possibility to construct combined stationary phases in a variety of ratios. This can be done by the serial coupling of column segments containing different homogeneous stationary phases whereby the intermediary length of each becomes the variable parameter of interest. Such an extended approach to the described 2-columns-in-series systems, was originally proposed by Nyiredy [227], [228]. Serially connecting several columns or column segments with different stationary phases is a flexible approach, since the column can subsequently also be dismantled and re-used for other separation problems.

In this way, an optimization workflow, similar to the PRISMA model for mobile phase optimization [98]–[101], can be developed for the prediction of retention and of selectivity under isocratic mobile phase conditions on such a coupled-column-in-series-system that contains multiple stationary phases. This approach is visualized in Figure 2.16.

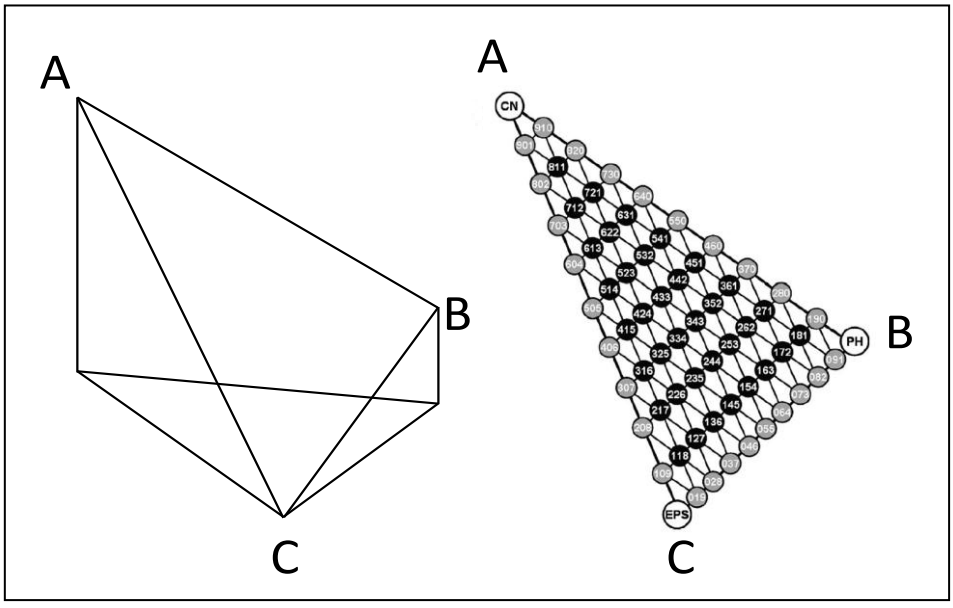

Fig. 2.16 The PRISMA model for stationary phase optimization.

Three different stationary phases are herewith present: A, B and C. The different stationary phases should ideally be orthogonal in terms of selectivity while still allowing the use of the same mobile phase. The lengths of the edges of the prisma, starting from the corners of the basis of the equilateral triangle, correspond with the polarity of the stationary phase. The top triangle, which is used for the stationary phase optimization, is not congruent with the basis triangle as different stationary phases are used. The corners of the top triangle correspond with the column composed of only one type of stationary phase. An example of a stationary phase combination can be represented in the triangle by combination of the volume fractions of the corners. A relation between the retention of a compound on the individual homogeneous stationary phases and the retention of this compound on such a serially connected stationary phase combination is essential to perform a systematic selectivity optimization towards an optimum in the triangle. The specific parameter to be optimized in this approach is the column length of each different stationary phase, represented in such a column-in-series system.

Practically, a first step in the process is the measurement of retention factors of the compounds of a sample on the individual stationary phases by means of preliminary scouting runs. One fixed mobile composition is thereby chosen arbitrarily and remains isocratic during the whole optimization process. Thereby, the applied column lengths can be chosen freely as the retention factor is independent of the column length.

The top triangle is considered as the solution space and is the collection of all possible stationary phase combinations. The size and shape of this solution space depends on the number of used stationary phases and on the unit length of a column segment in which the represented length of a stationary phase can vary in a column-in-series system. The length of such a unit column segment can be for example 1 cm.

Next, the solution space can be considered and screened. The retention factor of a compound on all combined stationary phase combinations *ABC* can be calculated by means of:

$$k_{ABC} = \frac{\phi_A k_A + \phi_B k_B + \phi_C k_C}{\phi_A + \phi_B + \phi_C} \qquad \text{(Eq. 2.32)}$$

k_A, k_B and k_C are the retention factors of a compound on the individual stationary phases *A, B* and *C*. Φ_A, Φ_B and Φ_C are the lengths of the 3 stationary phase in a combined stationary phase combination. Note that the order of the different serially connected stationary phases is irrelevant as this is an approach under isocratic conditions.

By this approach, the selectivity factors, retention factors and the retention times on every imaginable type of serial column combination can be calculated from the preliminary measurements of k on the (limited) number of pure phases. The critical peak pair can then be considered for each column segment combination and according to the value of the selectivity factors of these critical peak pairs, a ranking of the combinations can be made. Optionally, column combinations leading to excessive analysis times can be filtered out. Finally, the combination of stationary phases and associated column lengths, guaranteeing the highest selectivity factor for the critical peak pair of the sample within the desired analysis time, can be selected.

Practically, this approach can be performed with conventional commercially available columns as manufacturers offer their columns in different lengths which can be coupled with almost zero dead volume connectors [229]. Special kits, designed for this approach, are however developed by Bischoff Chromatography (Leonberg, Germany). A snapshot of this technology is shown in Figure 2.17. Herewith, the minimal length of a column segment unit of each stationary phase is 1 cm. Typically, lengths of up to 25 cm and larger can be made for any type of column combination. Note that this column length for the five available types of stationary phases already allow for the making of over 142 000 different kinds of combined columns. Therefore, statistically one has a high chance that in almost all separation applications a satisfactory solution can be found with this approach. The column segment technology allows coupling without creating any significant extra void volume and a user-friendly software package for the calculations based on equation 2.32 is available.

Fig. 2.17 Snapshot of column segments available in the POPLC® (Phase Optimized Liquid Chromatography) kit of Bischoff Chromatography (Leonberg, Germany) and the assemblage approach with small zero volume connectors. Note the availability of different column segment lengths and a color code for each type of individual stationary phase.

As an example, the separation of a test mixture on the kit developed by Bischoff Chromatography is shown in Fig. 2.18 [228]. A fixed mobile phase composition of 30 % acetonitrile and 70% water was thereby selected. The top three chromatograms represent the analyses on three individual stationary phases (CN: cyanopropyl, PH: phenyl, EPS (Enhanced Polar Selectivity): ODS with embedded polar groups). After measurement of the retention factors on the pure phases, the use of equation 2.32 for prediction of the chromatograms of all possible column combinations and selection of the optimal chromatogram, an optimal combination of 4 cm CN with 10 cm PH and 6 cm EPS was predicted. This combination is positioned in the solution space, defined by the top triangle shown in Fig. 2.16, and can be indicated with selectivity point P_S = 2-5-3 within this triangle, which represents the fractional presence of the individual stationary phases. As a result, a baseline separation is obtained (Fig 2.18: bottom).

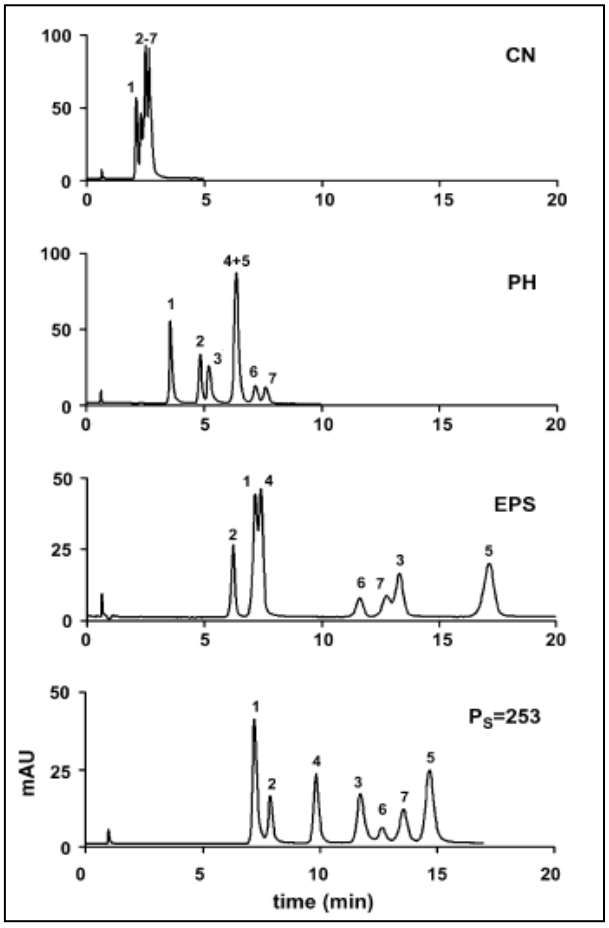

Fig. 2.18 Basic analyses on three individual stationary phases (CN,PH and EPS) and the separation obtained with the predicted optimal serially connected stationary phase combination (4 cm CN + 10 cm PH + 6 cm EPS, or represented by P_S = 253). Compounds: (1) methylparaben, (2) acetophenon, (3) ethylparaben, (4) dimethyl-phtalate, (5) 2,3-dimethyl-phenol, (6) methyl-benzoate and (7) anisol [228]**.**

A practical drawback of the PRISMA SOSLC approach is the limitation to isocratic analysis, making the approach inadequate for mixtures of compounds that differ significantly in polarity and hydrophobicity where a gradient analysis is required to obtain acceptable retention times.

To overcome these limitations, two approaches are investigated and described in Chapter 3 and Chapter 4 investigating the possibility to perform SOSLC in gradient elution mode. SOSLC approaches have recently also been reviewed [230], [231] and the potential for separating complex mixtures by combining orthogonal stationary phase selectivities, which are present more than often before, is underlined. Hereby, the reader is referred to the work described in Chapters 3 [232], 4 [233], 5 [234] and 6.

2.7.3.5 Variable column length strategy

Besides the described prediction approaches which are based on experimental design, "brute force" approaches which might be based on alternative principles and which can sometimes lead to a fit-for-purpose in a faster way, can exist as well. In this context, a recently developed approach is the variable column length strategy to expedite method development (VL-MD) [235]. It involves a generic method development strategy, useful when a sample with unknown composition is handled.

This strategy encloses several steps. In a first step, scouting runs are performed on short columns in order to screen different mobile phase and stationary phase conditions. Further on, the gradient profile is adapted to obtain a sufficiently wide elution window and acceptable retention factors for the first ($1 < k < 3$) and last ($10 < k < 15$) eluting compound. The influence of varying gradient conditions on the retention is hereby also evaluated. Out of these runs, the best run is selected and defined as the separation that depicts the largest number of separated compounds and the highest critical pair resolution. Optionally, the analysis time can be thereby also considered. The best run is then redone on different column lengths, including the maximal column length, to check the potential co-elution of compounds by comparing the widths of the peaks. As the width of the peaks that are separated with $R_S \geq 1$ increase in proportion to the square root of the column length, any deviation on this effect can indicate that multiple compounds co-elute under the same peak. In a next step the separation can then be fine tuned for the optimal column length for optimizing the selectivity for obtaining a satisfactory separation. This fine-tuning can be performed according to classic method development tools. The flow rate and the column length can hereby be adapted with the condition that the resolution of the critical pair is not reduced.

In principle, this strategy can be performed manually, however, in order for the approach to be of any practical benefits, the development of an automated system is thereby essential. The concept of

such an automatic column coupler (ACC) is shown in Figure 2.19. Note hereby that the selected stationary phases can be chosen arbitrarily by the chromatographist. This concept can in principle also lead to a fully automated overnight method development.

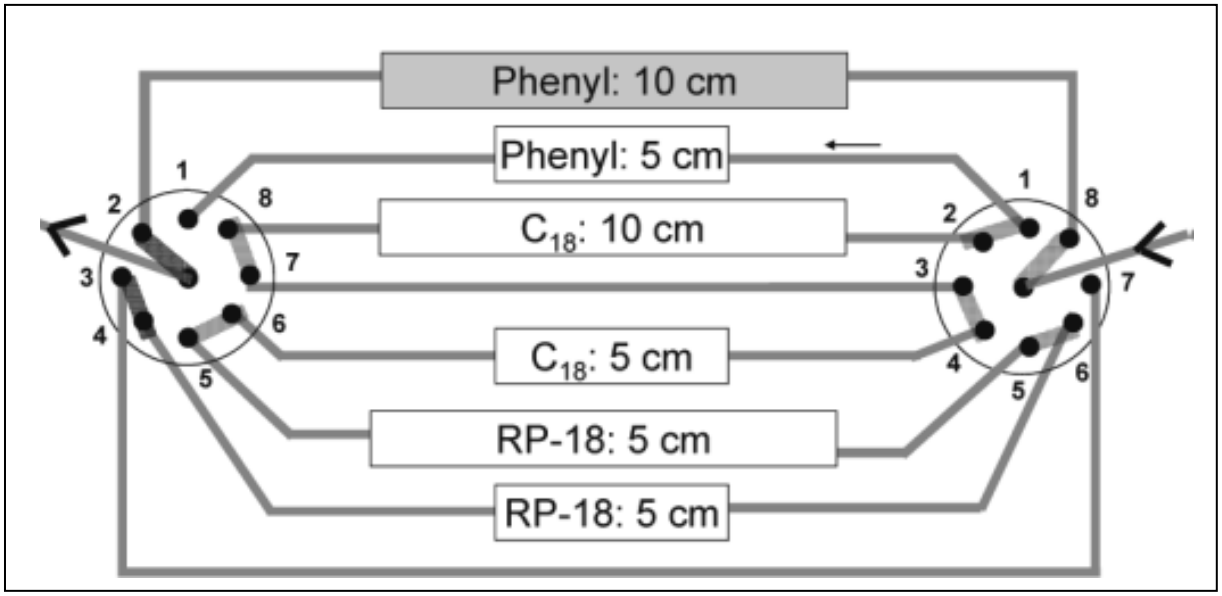

Fig. 2.19 Example of a set-up of an automated column coupler (ACC) which allows the coupling of columns of three different selectivities. The total column length can also be varied by means of the different valve combinations which are possible [235]**.**

2.7.4 Quantitative Structure-Retention Relationship (QSRR) modeling

A drawback of all the above described approaches is that they require the performing of a set of preliminary analyses before a solution for a problem can be found. An ideal optimization approach should require no preliminary experiments. In the past decades, efforts have been made to estimate or predict retention without any other knowledge besides the chemical structure of an analyte. Although these strategies have thus far not been combined with the above described SOSLC approach, there is an obvious elegance in combining in silico retention time predictions and the column combination approaches. It is therefore relevant to outline these principles before discussing the practical aspects and results of this thesis.

To predict a physicochemical property of a molecule, a relation between the chemical structure and the considered property can be investigated and modeled. Applied on chromatography, this would then be the relation between retention and the molecular structure of an analyte. Chromatography is a grateful technique for investigating such chemical structure-property relations. A large set of data of a large amount of diverse molecules can be obtained with a high level of precision and can be mutually related under constant experimental conditions, such as a fixed column and mobile phase

selection in an automated way. This field of study and investigation in chromatography is known as Quantitative Structure-Retention Relationships (QSRR) [196], [236]–[238].

To be able to model quantitative or mathematical relations, the molecular structure should be expressed or represented by means of molecular descriptors. A theoretical descriptor can be defined as the final result of a mathematical procedure. Hereby, the molecule is translated into a useful numeric value [239]. This stands in contrast with an experimental descriptor which is the result of a standardized experiment [240]. A large variety of theoretical descriptors can today be developed and assigned to an analyte. An important starting point for building QSRR models is the selection of a reduced set of statistically relevant descriptors, which lead to reliable modeled relationships. This is necessary as irrelevant descriptors can lead to statistic chance correlations [241]. QSRR models are typically built by multiple linear regressions, combinded with other chemometric tools, such as genetic algorithms [242], [243] or artificial neural networks [244]. First QSRR reports resulted from studies of quantitative structure-(biological) activity relationships (QSAR) [245], applied to the analysis of chromatographic data. The present concept and goals of QSRR studies are schematically presented in Figure 2.20 [238].

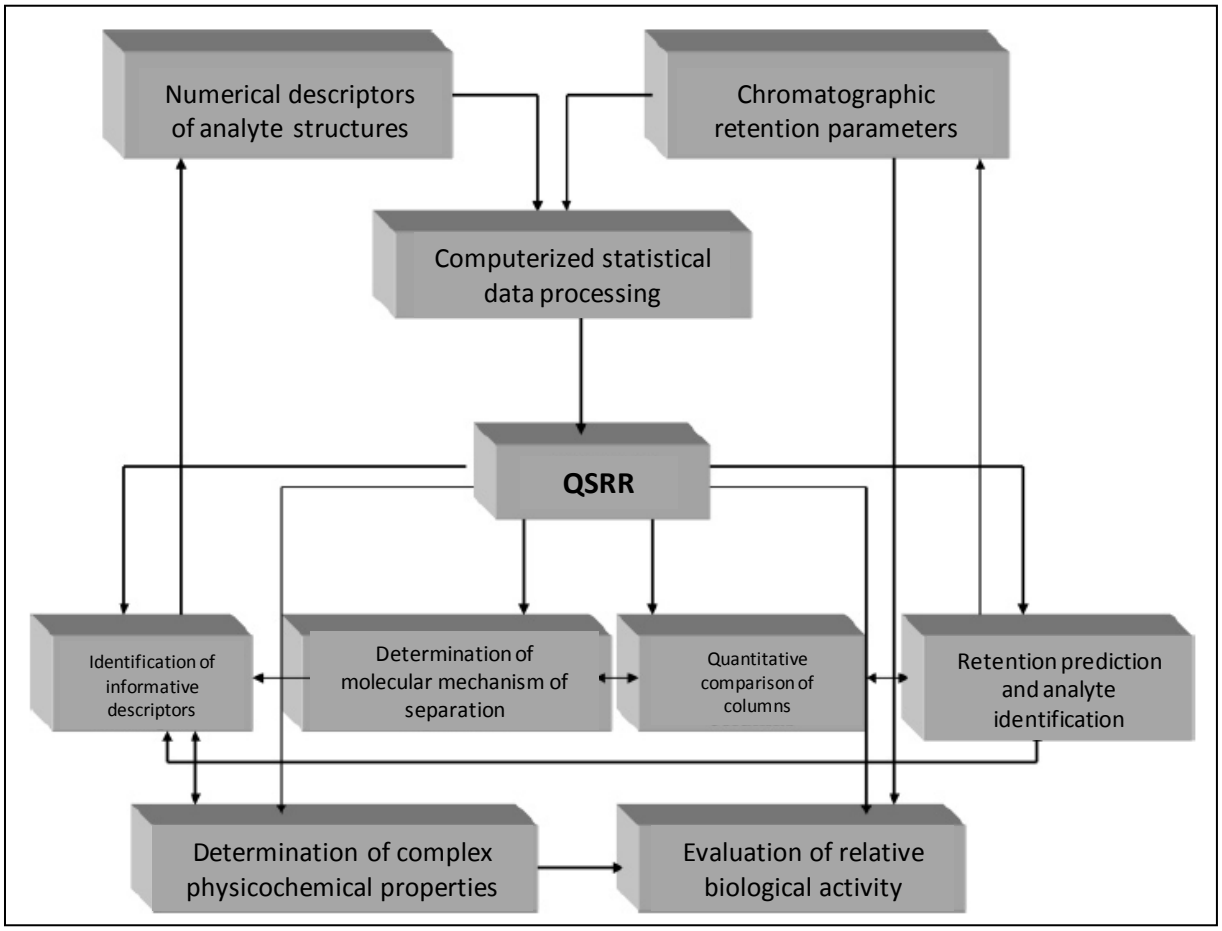

Fig 2.20 Overview of the methodology and application topics of QSRR studies [238]**.**

Practically, in order to be able to peform a QSRR study, a set of sufficiently large series of analytes and a set of their structural or molecular descriptors is required. By means of chemometrics and relationship modeling, their retention is characterized in terms of various analyte descriptors. If statistically significant and physically meaningful QSRR's are obtained, they can then be applied to [238]:

(A) identify the most useful (regarding properties) structural descriptors.

(B) predict retention for a new analyte.

(C) gain insight into molecular mechanism of retention and separation operating in a given chromatographic system.

(D) quantitatively compare separation properties of individual types of chromatographic columns.

(E) evaluate properties other than chromatographic physicochemical properties of analytes, such as lipophilicity and dissociation constants.

As mentioned, fixed chromatographic conditions allows the molecular structure of analyte to be thereby the single variable in the system. The composition of the input data set of training analytes plays an important role as well. A valid QSRR model requires an evaluation of the prediction performance of the model for new future analytes. Ideally, independent data by means of an external test set of new analytes should be used. However, a cross validation approach can be applied by extracting a small internal set of training analytes within the originally tested training set and by evaluating the reliability on this small internal set.

To predict the retention of a new analyte, the input data set of training analytes is used to search for similarity. Depending of the purpose of the research, the training data set is preferred to be rather versatile if a complete new type of analyte is handled, or rather dedicted to a certain class of compounds if a new analyte within this class is handled. For predicting or calculating the similarity between analytes, the similarity between relevant descriptors is considered. Multiple methods for similarity determination are described [240].

As mentioned thus far, QSRR approaches have only been used for retention time prediction on pure types of stationary phases. In the next chapters, various strategies are described for improvement of the "conventional" SOSLC strategy as developed by Nyiredy et al [227], [228].

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Chapter 3: Multiple step gradient analysis in stationary phase optimized selectivity LC for the analysis of complex mixtures

Summary

Stationary phase optimised selectivity liquid chromatography (SOSLC) is an approach to tune a given LC separation by combining different stationary phases in a multi-segment column set-up. SOSLC, its optimization procedure and algorithm made were originally developed only for use in isocratic HPLC. This is a severe limitation for the analysis of mixtures composed of components covering a broad hydrophobicity range. A strategy is described in this chapter to circumvent this limitation. The components of a mixture are divided into different groups according to hydrophobicity as elucidated by a gradient analysis on a C18 reversed-phase column. Each group separation is then individually optimized with a specific isocratic mobile phase composition using the original isocratic SOSLC strategy. The mobile phase composition thereby only differs in the percentage of organic modifier between the various groups. Finally, a combination of stationary phases that guarantees sufficient selectivity for all the groups is selected and the separation is performed by a multiple step gradient, whereby each level consists of the mobile phase composition applied for the SOSLC optimization of the individual groups. The multi step gradient approach is demonstrated through the analysis of a mixture of 27 steroids covering a wide range of hydrophobicity.

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3.1 Introduction

Selectivity optimization in LC is often performed by selecting an appropriate stationary phase followed by optimization of the mobile phase composition, as mentioned in section 2.7.1 [1–7]. Other approaches are the systematic modification of the chemical composition of the stationary phase [8, 9], the mixed-bed approach [10, 11] and the serial coupling of columns packed with different stationary phases [12–18]. More recently a strategy called stationary phase optimised selectivity liquid chromatography (SOSLC) was described (see section 2.7.3.4) [19, 20].

In SOSLC the stationary phase becomes a tunable parameter by connecting column segments of different stationary phases in series to obtain a desired column. The method is based on the PRISMA model for optimization of the mobile phase in LC [21]. An optimized column combination, giving the highest separation selectivity in the shortest analysis time, is thereby predicted based on the retention factors and selectivities obtained on the individual phases. The detailed optimization methodology of isocratic SOSLC is explained in section 2.7.3.4.

The whole concept including column segments and software (Phase optimized liquid chromatography – POPLC) is commercially available from Bischoff Chromatography (Leonberg, Germany). As mentioned, the main drawback of the method is that it is limited to isocratic analysis. For complex mixtures with compounds that differ significantly in polarity and hydrophobicity, the use of a fixed mobile phase composition will not allow the elution of all solutes in an acceptable analysis time. Late eluting peaks might suffer from excessive peak broadening, decreasing compound sensitivity [22, 23].

In conventional LC, gradient analysis is commonly applied leading to faster analysis, improved signal to noise ratios and reduced peakwidths compared to isocratic analysis. In order to extend the possibilities of SOSLC, a gradient compatible approach was developed by means of a multiple step gradient [24], which is presented in this chapter. Step gradient SOSLC in combination with mass spectrometric detection was later applied by Gostomski et al. [25] for the sensitive detection of impurities in synthetic thyroid hormones. In this chapter, The step gradient SOSLC method is illustrated with the separation of 27 steroids using the SOSLC step gradient procedure. Isocratic separations of 5 steroids by means of SOSLC has also been described by Kuehnle et al. [26].

3.2 Experimental

3.2.1 Materials

Triamcinolone, estriol, prednisolone, cortisone, methylprednisolone, dexamethasone, fluoxymesterone, triamcinolone acetonide, cortexolone, alfasone, estradiol, ethynylestradiol, estrone, testosterone, methyltestosterone, norethisterone acetate, megestrol acetate, medroxyprogesterone acetate, testosterone propionate, progesterone, nortestosterone, testosterone phenylpropionate and the HPLC solvents were purchased from Sigma-Aldrich (Bornem, Belgium). Methylboldenone, norclostebol acetate and chlorotestosterone acetate were purchased from Steraloids (Newport, RI, USA). Delmadinone and algesterone were purchased from Finechemie (Chongqing, China).

Stock solutions of 27 steroids with a concentration of 1 mg mL^{-1} were prepared in acetonitrile. The mixture of the 27 steroids and the individual steroid samples were prepared by diluting the stock solutions with 50/50 water/acetonitrile (ACN) to a final concentration of 30 µg mL^{-1}.

3.2.2 LC Separations

A POPLC Basic Kit 250-5 was purchased from Bischoff Chromatography (Leonberg, Germany). This kit contains following stationary phases: ProntoSIL C18 SH 2 (which is a classic C18 stationary phase), ProntoSIL C18 EPS 2 (which is a C18 phase with embedded polar amide groups), ProntoSIL Phenyl 2, ProntoSIL CN 2 and ProntoSIL C30. For each of these stationary phases, column segments of 10, 20, 40 (2x), 60 and 80 mm were available. These stationary phases all have particle sizes of 5 µm and the columns segments have internal diameters of 3 mm. With additional POPLC connecting pieces robust columns composed of combinations of these column segments could be assembled. All 5 stationary phases were applied in this study. A Hewlett Packard 1050 system equipped with a DAD-detector (Agilent, Waldbronn, Germany) was used for the LC analyses. The mobile phases were composed of water and ACN only. Detection was performed at 230 nm. The injected volumes were 5 µL and the flow rate was always 0.5 mL min^{-1}. Chemstation software (Agilent, Waldbronn, Germany) was used for data collection. Next to the POPLC software which was used for the initial experiments, the algorithms were also rewritten in Microsoft Excel and Visual Basic to allow automatic searching of the common stationary phase combinations proposed for the different groups by the algorithm. Conventional gradient analysis of the steroid mixture was performed on a 15 cm x 4.6 mm ID x 3.5

µm dp particles Zorbax Stable Bond C18 (Agilent Technologies, Brussels, Belgium). The column was operated at 1 mL min^{-1} in a gradient from 90/10 water/ACN to 10/90 water/ACN in 30 min.

3.3 Results and discussion

At present, gradient reversed-phase LC is by far the most applied method in pharmaceutical analysis. Figure 3.1 shows the chromatogram of the steroid mixture in gradient analysis. The critical pairs are peaks 8/9, 14/15 and 18/19 and changing the mobile phase composition hardly improved the separation (data not shown). Other stationary phases could be selected to improve the separation of the critical pairs but other compounds may then overlap. The chromatogram in Figure 3.1 was split in 3 parts according to the hydrophobicity of the solutes (Table 3.1) and the 3 parts were analysed in the isocratic elution mode using SOSLC. Compounds 1 to 10 allocated in the first group were analysed with 70/30 water/ACN which is the average amount of organic modifier to elute compounds 1 to 10 in Figure 3.1. The separation of compounds 11 to 20 (second group) was optimized by using 55/45 water/ACN and group 3, composed of solutes 21 to 27 and in which no co-elution occurred, was analysed at 40/60 water/ACN.

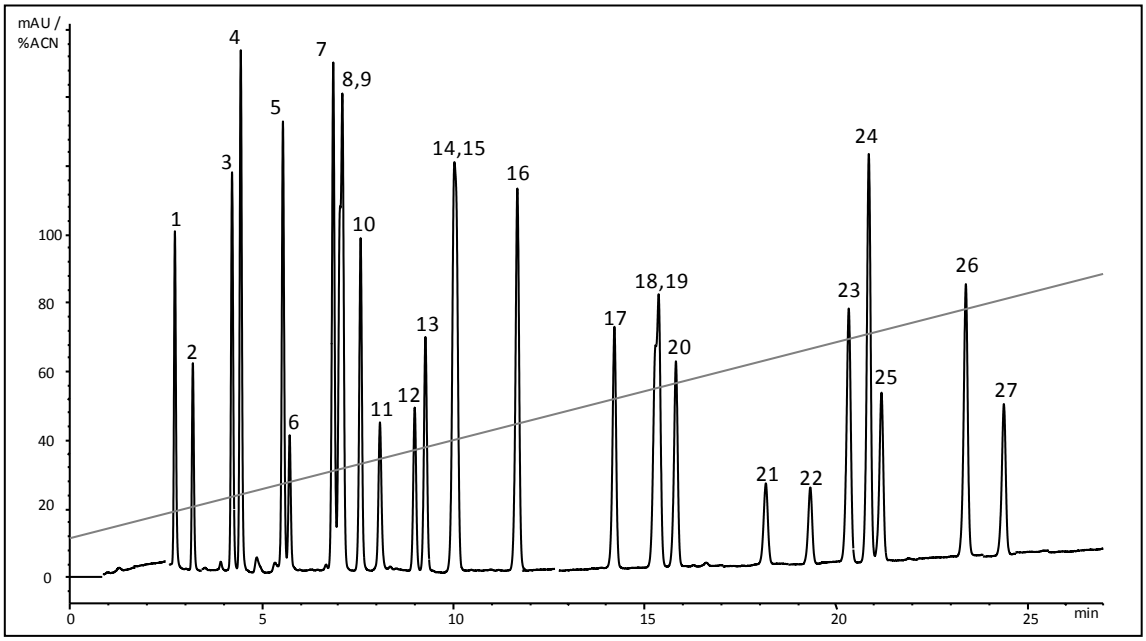

Fig. 3.1 Gradient analysis of the steroid mixture on a 15 cm x 4.6 mm ID x 3.5 µm d_p particles Zorbax Stable Bond C18. Conditions: see text. Peak numbering: see Table 3.1

Table 3.1 Classification of 27 steroids into 3 groups according to their hydrophobicity based on an analysis on a C_{18} column.

Group 1	Group 2	Group 3
1) triamcinolone	11) estradiol	21) norclostebol acetate
2) estriol	12) ethynylestradiol	22) chlorotestosterone acetate
3) prednisolone	13) estrone	23) testosterone propionate
4) cortisone	14) testosterone	24) progesterone
5) methylprednisolone	15) methylboldenone	25) algesterone
6) dexamethasone	16) methyltestosterone	26) nortestosterone
7) fluoxymesterone	17) delmadinone	27) testosterone phenylpropionate
8) triamcinolone acetonide	18) norethisterone acetate	
9) cortexolone	19) megestrol acetate	
10) alfasone	20) medroxyprogesterone acetate	

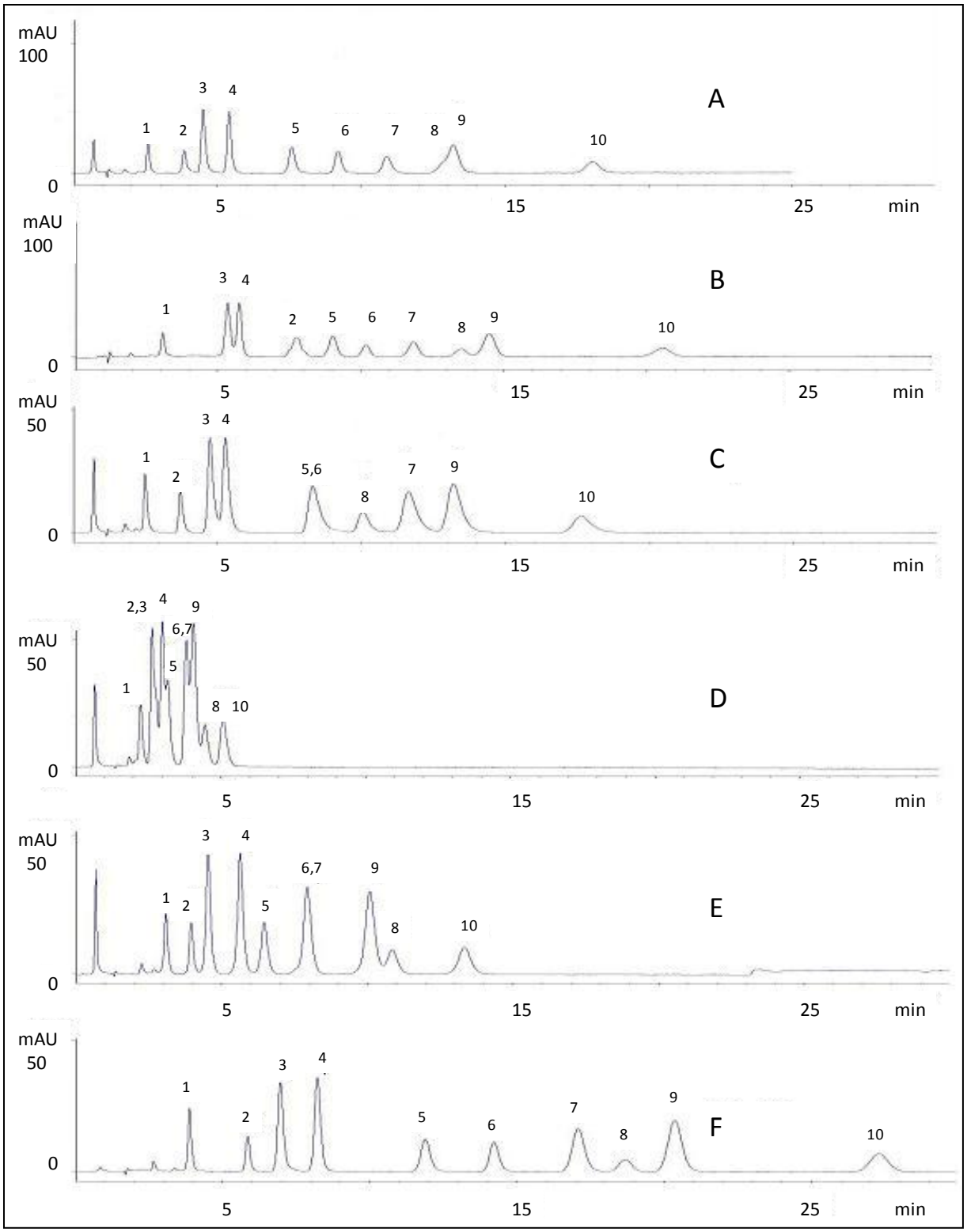

Fig. 3.2 Isocratic analysis of group 1 on 100 mm column lengths of A. C18 SH2, B. C18 EPS 2, C. C30, D. CN 2 and E. Phenyl 2. F. Experimental separation of group 1 on the predicted POPLC combination 30 mm C30 + 120 mm C18 SH 2 (see Fig. 3.3). Mobile phase composition: water/ACN 70/30 v/v. Peak numbering: see Table 3.1

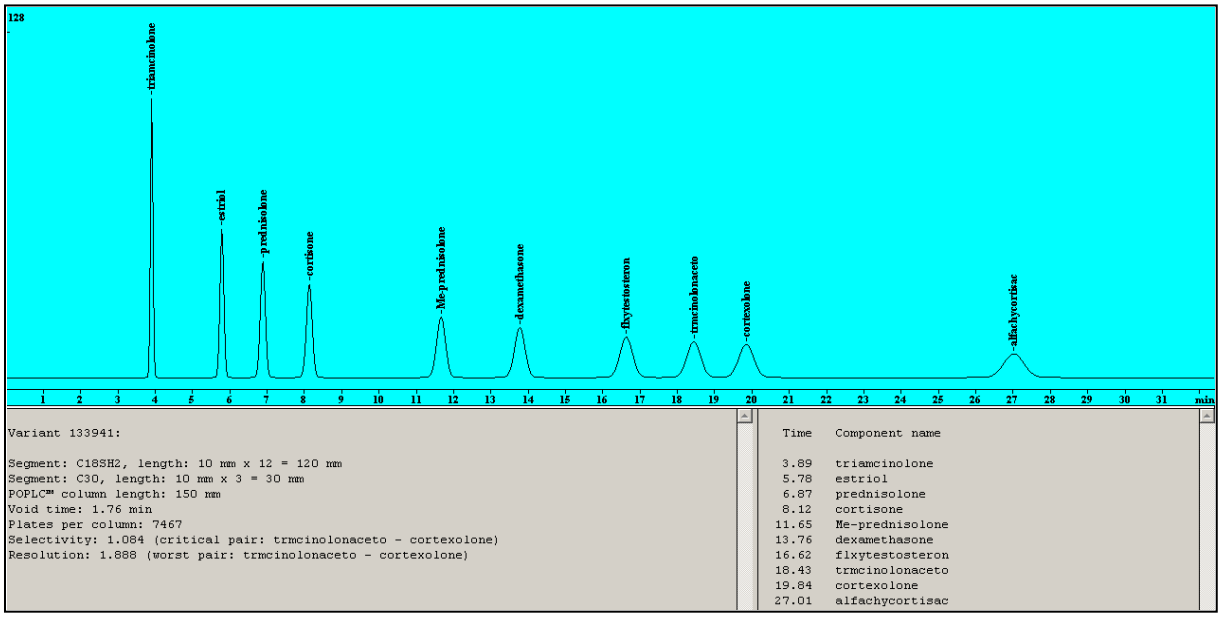

Fig. 3.3. Screenshot of the predicted POPLC chromatogram of group 1.

The chromatograms obtained on the 5 individual columns (10 cm in length) for group 1 are shown in Figure 3.2. As expected, on the C18 SH2 stationary phase (Fig. 3.2a), a similar profile was obtained as in Figure 3.1 and peaks 8 and 9 co-elute. An improved separation was noted on the C18 EPS 2 stationary phase (Fig. 3.2b) but peaks 3 and 4 start to be a critical pair and the column should be longer to provide baseline resolution of 3 and 4. On the C30 stationary phase (Fig. 3.2c), peaks 5 and 6 co-elute. Fig. 3.2d shows that CN 2 is not an appropriate stationary phase for steroid separation while on the Phenyl 2 stationary phase (Fig. 3.2e), peaks 6 and 7 co-elute. The chromatographic data of Fig. 3.2a–e were introduced in the software (POPLC) asking for the best separation in 30 min. The simulated chromatogram is shown in Figure 3.3 and the proposed stationary phase combination is 120 mm C18 SH 2 and 30 mm C30. The column was assembled and the experimental outcome is shown in Figure 3.2f. The similarity between the predicted and real chromatograms is remarkable illustrating the power of stationary phase optimization in isocratic LC.

The same optimization procedure was repeated for isocratic analysis of groups 2 and 3 using 55/45 and 40/60 water/ACN, respectively. Separation of group 2, containing the unresolved pairs 14/15 and 18/19, was most challenging and complete resolution, repeating the procedure described for group 1, could only be obtained in 40 min on the combination 10 mm C18 SH2, 40 mm C18 EPS, 60 mm Phenyl 2 and 20 mm C30. The separation of group 3 was not critical (see Fig. 3.1) and baseline resolution was obtained on 10 cm C18 SH2. All other phases resulted in co-elutions. However, the highest resolution in 30 min was achieved on 140 mm C18 SH2 plus 10 mm C18 while co-elutions

occurred on all other stationary phases. From the isocratic analyses, we can conclude that the optimized stationary phase combinations for the 3 groups are different. Considering that 142,505 column combinations can be obtained on a column of maximum 250 mm, the probability of obtaining the same optimal stationary phase combination which is the same for all groups, indeed, is extremely low. The next step is to find column combinations that are providing sufficient resolution in a given time. Based on the analysis time of each group (30 + 40 + 30 min), a total analysis time of 120 min was selected as maximum allowable analysis time. A ranking of all suitable stationary phase combinations was subsequently made for each group in decreasing order of the selectivity factor of the critical peak pair of each group. Part of this outcome is shown in Figure 3.4. Because it appeared that under the given experimental conditions no common column combination could be found for a set selectivity of 1.6 for the most critical pair, the value of the latter was decreased until a common column composition could be found for all groups. A reduction of the selectivity criterion leads to an increase in the suitable column combinations enhancing the probability to find a common phase. The first combination that could be found in this way is consisting of 20 mm C30, 120 mm C18 EPS 2 and 30 mm C18 SH2. The selectivity of the critical pair was therefore lowered to 1.2. This group comparison procedure could not be done with the original POPLC software and a program, making exactly the same predictions, was therefore written in Visual Basic (Excel).

Group 1					**Group 2**					**Group 3**				
A	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**
0	0	11	7	0	0	7	5	0	11	**3**	**12**	**2**	**0**	**0**
3	**12**	**2**	**0**	**0**	0	9	4	0	9	14	0	1	0	0
1	0	12	2	0	3	14	1	0	2	10	6	0	4	1
0	10	0	7	4	**3**	**12**	**2**	**0**	**0**	12	7	0	1	1
4	13	3	5	0	0	15	1	3	4	7	7	0	3	2
…	…	…	…	…	…	…	…	…	…	…	…	…	…	…

Fig. 3.4 Part of the outcome of the predictions obtained by Visual Basic (Excel).

The measured retention times are listed in Table 3.2.

This column combination was assembled for the analysis of the steroid mixture and a step gradient, corresponding to the mobile phase compositions used for the optimisation of the individual groups, was applied. The resulting chromatogram is shown in Figure 3.5.

When the last compound of group 1 eluted, the mobile phase composition was changed in 0.1 min to the second isocratic level (applied for the optimization of group 2) and when the last compound of the second group eluted, the mobile phase composition was changed in the same way to the third isocratic level (applied for the optimization of group 3). By performing the experiment in this way the migrated distances of the analyte bands of group 2 through a column are very short under the mobile phase conditions of group 1. Therefore the elution (order) of group 2 is almost unaffected by the mobile phase composition of group 1 during the first stage of the total optimized chromatographic run. The total retention time of the compounds from group 2 can therefore roughly be obtained through the sum of the total analysis time of group 1 (30 min), the predicted retention time (at 45% acetonitrile) for the compounds of group 2 and the system void time. The same reasoning is valid for group 3.

Finally, all 27 steroids are separated what could not be obtained on a single stationary phase in a continuous gradient analysis (Fig. 3.1).

Table 3.2 Predicted retention times on a common stationary phase composed of 2 cm C30, 12 cm C18 EPS 2 and 3 cm C18 SH 2.

Compound	t_R (min)
triamcinolone (1)	4.83
estriol (2)	10.73
prednisolone (3)	8.58
cortisone (4)	9.27
methylprednisolone (5)	14.49
dexamethasone (6)	16.32
fluoxymesterone (7)	19.36
triamcinolone acetonide (8)	21.05
cortexolone (9)	22.90
alfasone (10)	31.24
estradiol (11)	48.38
ethynylestradiol (12)	53.52
estrone (13)	49.40
testosterone (14)	40.97
methylboldenone (15)	35.56
methyltestosterone (16)	43.80
delmadinone (17)	59.09
nortethisterone acetate (18)	65.15
megestrol acetate (19)	63.58
medroxyprogesterone acetate (20)	67.15
norclostebol acetate (21)	81.43
chlorotestosterone acetate (22)	85.25
testosteron propionate (23)	88.46
progesterone (24)	110.96
algesterone (25)	86.72
nortestosterone (26)	108.36
testosteronphenyl propionate (27)	120.88

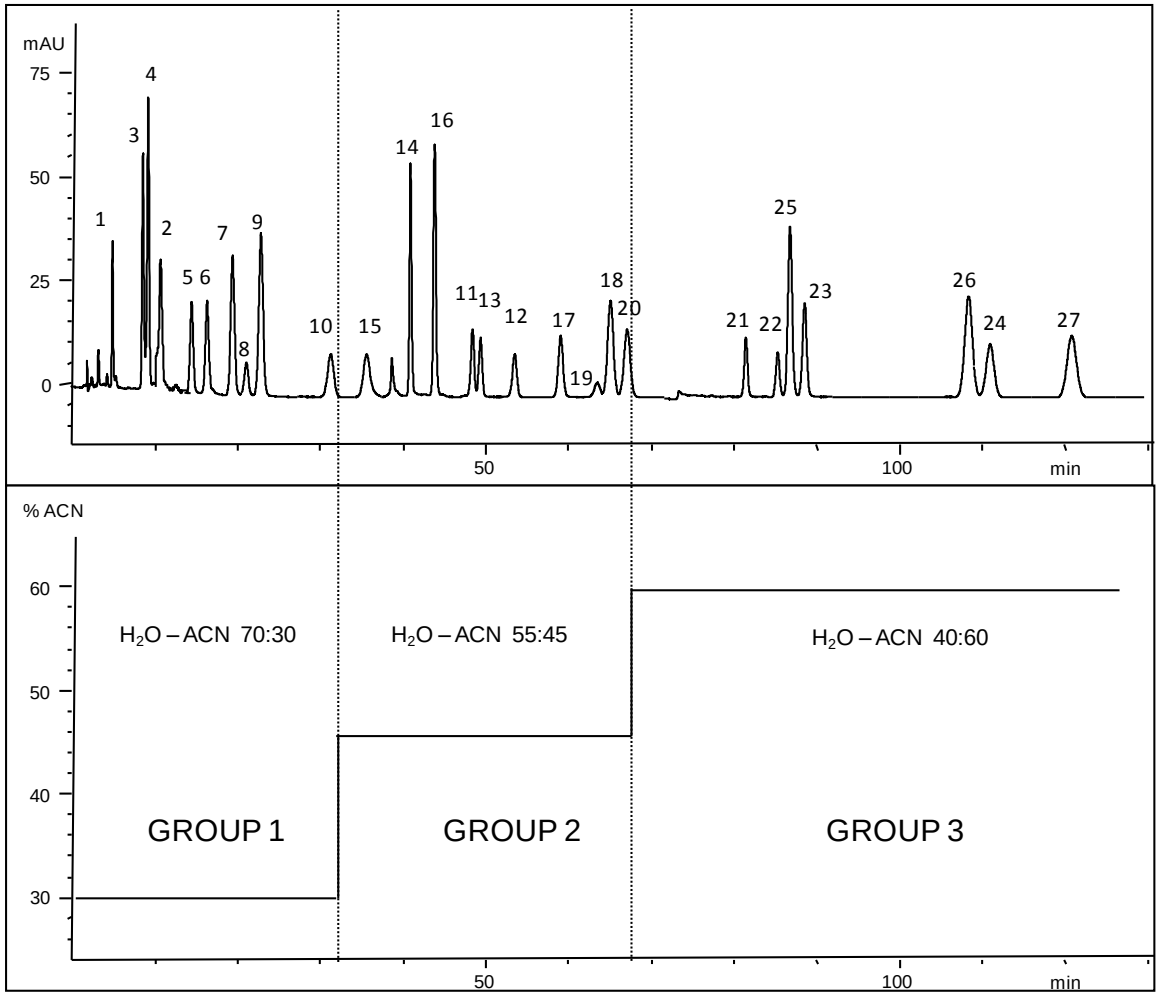

Fig. 3.5 Separation of the steroid mixture by step gradient on 30 mm C18 SH 2 + 120 mm C18 EPS 2 + 20 mm C30. Peak numbering: see Table 3.1. Conditions: see text. Peak numbering: see Table 3.1

3.4 Conclusions

The possibilities of SOSLC have been extended by developing a step gradient strategy. The compounds of a complex mixture are first classified into groups according to increasing hydrophobicity on a standard C18 column. Each group then undergoes the SOSLC optimization procedure for isocratic analysis. Finally, a common stationary phase combination guaranteeing sufficient selectivity for each group is selected. The optimized separation is then performed with a multiple step gradient whereby each plateau in the mobile phase composition graph corresponds to the mobile phase compositions used for the optimization of each group.

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Chapter 4 Stationary phase optimized selectivity liquid chromatography: development of a linear gradient algorithm

Summary

Stationary-phase optimized selectivity liquid chromatography (SOSLC) is a tool in reversed-phase LC (RP-LC) to optimize the selectivity for a given separation by combining stationary phases in a multi-segment column. The presently (commercially) available SOSLC optimization procedure and algorithm are only applicable to isocratic analyses. Step gradient SOSLC has been developed and described in Section 3 but this is maybe not ultimately elegant for the analysis of complex mixtures composed of components covering a broad hydrophobicity range as exact retention times are thereby not predicted. Therefore in this chapter, a linear gradient prediction algorithm has been developed allowing to apply SOSLC as a generic RP-LC optimization method. The algorithm allows operation in isocratic, step-wise and linear gradient run modes. The features of SOSLC in linear gradient mode are demonstrated by means of a mixture of 13 steroids, whereby baseline separation is accurately predicted and experimentally demonstrated.

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4.1 Introduction

Despite the wide choice of optimization parameters, selectivity can sometimes remain elusive and in such cases, selection of an alternative stationary phase is necessary. The selection rationale for an alternative stationary phase is not standardized, and often either the chromatographers experience and preference or literature precedence primarily influence this choice with trial and error being the second most common approach [1-7]. Many attempts have been made by researchers in the literature to propose strategies which may facilitate stationary phase selection including for example stationary phase classification models [8-13], however, these are presently somewhat diverse and certainly not standardized. Hereby, the problem is the sheer number of RP-stationary phases commercially available which increases on an annual basis without offering dramatic alternative selectivities [14]. This fact is somewhat disguised by the fact that manufacturers do not use a harmonized approach for explaining the selectivity (i.e. a standard set of test compounds).

One innovative approach to circumvent trial and error experiments is through employment of stationary-phase optimized selectivity liquid chromatography (SOSLC) whereby the stationary phase becomes a tunable parameter by connecting column segments, with variable lengths, of different stationary phases [15-21]. An optimization procedure and algorithm based on the PRISMA model for optimization of the mobile phase in LC [22] has been developed to apply this strategy under isocratic conditions [23-24]. A practical drawback with the rise of this method and the commercial product, was the limitation to isocratic analysis, making the approach inadequate for mixtures of compounds that differ significantly in polarity and hydrophobicity where a gradient analysis is needed for obtaining acceptable retention times. Moreover, late eluting compounds may suffer from excessive peak broadening and decreasing compound detectability [25-26].

To overcome these limitations, in the precedent chapter a multiple step gradient method was developed and described [27]. Hereby, an arbitrary classification of the compounds of a mixture is made into a few groups according to their relative polarity/hydrophobicity. Each group is then handled with the classic isocratic SOSLC procedure and a common column segment combination that guarantees sufficient selectivity within all groups is obtained. The mobile phase for the optimized column is a multiple step gradient profile consisting of a sequence of isocratic levels.

Although useful, this step gradient method is characterized by some fundamental limitations. Firstly, in gradient analysis, the order in which the different stationary phases are connected is expected to influence retention and selectivity. As the method is still based on Eq. 2.32 (see Chapter 2) and as this equation does not give any restrictions to the order of the stationary phases in a combined column,

this method only allows the prediction of a multi-segment column that guarantees sufficient selectivity and retention times can only be roughly estimated. Secondly, the classification of the compounds into groups is arbitrary and a more practical approach is mandatory for routine use of SOS-LC.

A general prediction algorithm, based on numerical integration, and optimization method – described in this chapter – was developed that provides for a more flexible application of SOSLC by enabling the system to be utilized with a linear gradient mode. Furthermore, arbitrary decisions about sample components are eliminated and accurate predictions of all retention times and selectivity factors can be obtained. The use of discontinuous or numerical integration approaches is currently well established in the chromatographic field [28-43].

4.2 Experimental

4.2.1 Materials

Acetophenone, aniline, caffeine, phenol, pyridine, benzene, propylparaben, toluene, prednisolone, methylprednisolone, cortisone, triamcinolone acetonide, cortexolone, methylboldenone, estradiol, testosterone, methyltestosterone, testosteron propionate, testosterone phenylpropionate, progesterone, nortestosterone and the HPLC solvents were purchased from Sigma-Aldrich (Bornem, Belgium). Stock solutions of all compounds with a concentration of 1 mg mL^{-1} were prepared in acetonitrile. A test mixture and individual samples of the first 8 compounds as well as the mixture of the 13 steroids and the individual steroid samples were prepared by diluting the stock solutions with 50/50 water/acetonitrile (ACN) to a final concentration of 30 µg mL^{-1}.

4.2.2 Methods

A POPLC Basic Kit 250-5 from Bischoff Chromatography (Leonberg, Germany) was used. The kit contains the following stationary phases: ProntoSIL C18 SH 2 (a classic C18 stationary phase), ProntoSIL C18 EPS 2 (a C18 phase with embedded polar amide groups), ProntoSIL Phenyl 2, ProntoSIL C30 and ProntoSIL CN 2. For each of these phases, column segments of 10, 20, 40 (2 x), 60 and 80 mm are available. The stationary phases all have a particle size of 5 µm and the column segments have an internal diameter of 3 mm. With additional POPLC connecting pieces, robust columns composed of combinations of these column segments can be assembled. A Hewlett Packard 1050 HPLC system equipped with DAD-detector was used (Agilent Technologies, Waldbronn, Germany). The mobile phases were composed of water and ACN only. Detection was performed at 210 nm for

the test mixture and at 230 nm for the steroid mixture. The injected volumes were 5 µL and the flow rate was always set at 1 mL min^{-1}. Chemstation software (Agilent Technologies) was used for retention data collection.

4.2.3 Safety consideration

All conventional safety concerns when working with HPLC were taken into consideration. This included avoiding contact with acetonitrile by the use of appropriate gloves and safety goggles at all times. Particular care was taken when preparing and analyzing the steroids samples because of their known endocrine disrupting behaviour. All solvent and sample waste was considered as chemically hazardous and was disposed of in dedicated containers for further specialized treatment.

4.3 Results and discussion

The first step in developing an SOSLC method for linear gradient, is modeling of the retention behaviour of a compound as a function of the percentage of the organic modifier concentration φ [44,45]. This is well documented in the literature and the quadratic relationship between *ln(k)* and φ (Eq. 4.1), developed by Schoenmakers et al. was applied as it is one of the most accepted models [46-47].

$$ln(k) = a\varphi^2 + b\varphi + c \quad \text{(Eq 4.1)}$$

a, *b* and *c* are thereby experimental coefficients and at least 3 isocratic analyses at different modifier concentrations φ are needed to determine these coefficients. Eq. 4.1 is necessary for an algorithm that predicts retention times under linear gradient conditions. Note that when working in a small enough gradient range, the retention behavior of some compounds can be described accurately enough with a variant of Eq. 4.1:

$$ln(k) = b\varphi + c \quad \text{(Eq. 4.2)}$$

This also offers an elegant solution to the possible and statistical problem of overfitting with Eq. 4.1, because $a\varphi^2$ can be equalized to 0 and easily be eliminated in this way. However, the quadratic model remains generally valid when the minimum of 3 data points are equidistantly positioned over the measured range (small or large) of φ, as the quadratic equation is sufficiently proven to describe physically the retention behavior in the case of reversed phase liquid chromatography. An alternative solution to check or increase the reliability of the model, is by simply adding more measured data points.

Multiple approaches and algorithms were developed in the past in order to predict retention times under linear gradient conditions [28-51]. In all approaches, other parameters and aspects beside Eq. 4.1 have to be considered. A mandatory parameter is thereby the dwell time, t_{dwell}. This is the time needed for the front of a changed mobile phase composition to reach the column inlet. t_{dwell} can easily be measured by connecting the tubing, that normally is connected to the column inlet, directly

to the UV detector. t_{dwell} is then the time measured between the initialising of a step gradient in the LC pump and the subsequent deviation in the UV signal baseline.

In this study, a so called discontinuous prediction algorithm based on numerical integration was developed. A linear gradient is hereby considered as the sequence of small isocratic stages. This discontinuous approach allows the determination of the intermediate migrated distance through the column at each time point during the analysis. This is a useful approach when coupled columns, containing different stationary phases, are considered. The concept is clarified in Figure 4.1.

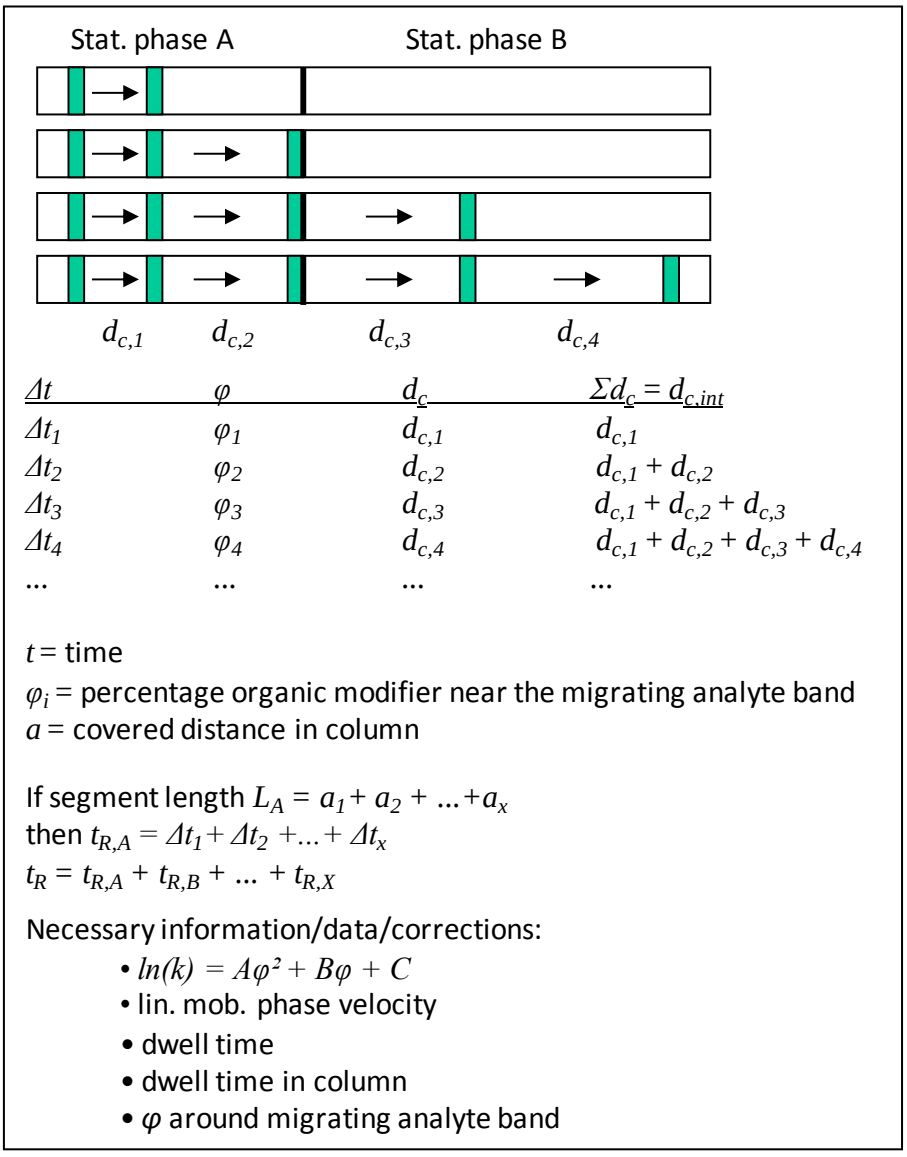

Fig. 4.1 Concept of the prediction algorithm for retention times under linear gradient conditions on combined column segments

If an analyte band passes the end of a first stationary phase, the algorithm allows the substitution of Eq. 4.1 of the analyte on the first stationary phase into Eq. 4.1 for the same analyte on the second stationary phase, for which other *a, b* and *c* values are determined.

The algorithm further requires the knowledge of the continuously increasing φ around the migrating analyte at each time during the analysis. Consequently, this requires an extra correction in the algorithm, resulting from the continuously increasing dwell time in the column. As an analyte band is positioned somewhere in the column, the time needed for a new mobile phase composition to reach this analyte band is the sum of the dwell time of the instrument, t_{dwell}, and the dwell time in the column, $t_{dwell,column}$. For this correction to be inserted into the algorithm, only the linear velocity of the mobile phase, v_0, has to be known. v_0 can be determined by means of Eq. 4.3.

$$v_0 = \frac{L}{t_0} \qquad \text{(Eq. 4.3)}$$

L is the column length and t_0 is the void time. Consideration of these accumulated corrections leads to the following prediction algorithm. The profile of the linear gradient can be described as follows:

$$\varphi = \varphi_0 + qt \qquad \text{(Eq. 4.4)}$$

where φ_0 is the initial organic modifier concentration and q is the slope of the linear gradient profile. t is the independent variable time during the analysis. The moment a gradient is initialised in the LC pump, the compounds of an injected sample are still subordinate to isocratic migration until the instrument dwell time t_{dwell} has passed. φ is thus for this part of the linear gradient analysis considered as a constant and equal to φ_0. k can be derivated by means of Eq. 4.1. The corresponding retention time t_t can be obtained by means of the definition of k:

$$t_t = kt_0 + t_0 \qquad \text{(Eq. 4.5)}$$

where t_0 is the void time that corresponds with a column with its specific stationary phase and length *L* that is used during the preliminary measurements in order to measure the *a*, *b* and *c* values. The covered distance of an analyte band in a certain time interval Δt, d_c, can be calculated by dividing *L* through t_t.

$$d_c = \frac{L}{pt_t} \quad \text{(Eq. 4.6)}$$

p is an arbitrary parameter which has to be selected such that the resulting Δt is smaller than the time required by the LC-pump to change the composition of the mobile phase during a linear gradient run. For example, if *p* is put equal to 1, then Δt is 1 second. Note that a too small *p* will lead to increased computing time.

$$\Delta t = pt_t \quad \text{(Eq. 4.7)}$$

The total covered distance during the dwell time of the gradient, $d_{c,dwell}$, can be calculated by summation.

$$d_{c,dwell} = \sum_{i=1}^{n} d_{c,\Delta t_i} \quad \text{(Eq. 4.8)}$$

$$n = \frac{t_{dwell}}{\Delta t} \quad \text{(Eq. 4.9)}$$

Δt may be slightly adapted by changing *p* in Eq. 4.7 in order to make *n* an integer. d_c is a constant for all Δt_i in this isocratic migration part of the gradient, if considering one uniform stationary phase. Note that in the case of coupled stationary phases and when a very fast migrating analyte band already passes the first stationary phase during the dwell time, d_c of this compound on the first stationary phase has to be replaced then by d_c, valid for the next stationary phase. The intermediary

passed analysis time, $t_{r,dwell,int}$, corresponding to the intermediary covered distance of an analyte during the dwell time t_{dwell}, can be obtained by the analogue summation of Δt_i.

$$t_{r,dwell,\mathrm{int}} = \sum_{i=1} \Delta t_i \qquad \text{(Eq. 4.10)}$$

When t_{dwell} has passed, the equations above have to be adapted. The changing of φ, the continuously increasing dwell time in the column $t_{dwell,column}$ and the consequent fact that an analyte band remains migrating isocratically for a while after t_{dwell}, has to be kept in mind. The determination of the covered distance of an analyte band through the column in a time interval Δt_j, after the dwell time t_{dwell} has passed, can be calculated as follows. The linear velocity of the mobile phase is determined by means of Eq. 4.3. The increased dwell time in the column at Δt_j, $t_{dwell,column,\Delta t_j}$, can be calculated by dividing the intermediary covered distance of the analyte band over the column, $d_{c,int}$, by the linear velocity of the mobile phase (Eq. 4.11).

$$t_{dwell,column,\Delta t_j} = \frac{d_{c,\mathrm{int}}}{v_0} \qquad \text{(Eq. 4.11)}$$

The organic modifier concentration that encounters the migrating analyte band at that time interval Δt_j is determined by a corrected version of Eq. 4.4.

$$\varphi = \varphi_0 + q(t_{r,\mathrm{int}} - t_{dwell} - t_{dwell,column,\Delta t_j}) \qquad \text{(Eq. 4.12)}$$

$t_{r,int}$ is the intermediary retention time of the analyte band that can continuously be calculated in the same way as Eq. 4.10 as it also the summation of all the previous time intervals Δt.

Knowing φ near the analyte band at Δt_j by means of Eq. 4.12 and using it with Eq. 4.1 and Eq. 4.5, leads to Eq. 4.6 which describes the covered distance of the analyte band during Δt_j. The

intermediary covered distance through the column can be calculated by summation of the covered distances corresponding with all the previous time intervals *Δt*.

$$d_{c,\mathrm{int}} = d_{c,dwell} + \sum_{j=n+1}^{m} d_{c,\Delta t_j} \qquad \text{(Eq. 4.13)}$$

Hereby, *n* is derived from Eq. 4.9. When the iterative increasing $d_{c,int}$ becomes equal to the total column length, the corresponding final retention time t_r of the compound is equal to the summation of all the time intervals *Δt* (Eq. 4.14).

$$t_r = \sum_{i=1}^{n} \Delta t_i + \sum_{j=n+1}^{m} \Delta t_j \qquad \text{(Eq. 4.14)}$$

Note again, in the case of coupled stationary phases when one stationary phase ends and is followed by another stationary phase in a column, the *a*, *b* and *c* terms have to be replaced at the right time in the calculation process.

From the predicted linear gradient and retention times for all compounds in a mixture for all possible column segment combinations, a chromatographic response function such as the selectivity factor or difference between consecutive retention times can be calculated. The whole prediction process can be performed either for a fixed gradient profile or if necessary, it can be repeated for multiple gradient profiles. Finally, all possible column combinations are ranked according to their highest value of the applied chromatographic response function for the most critical peak pair in the sample mixture. The highest ranked column segment combination can then be selected and assembled as the optimal column. Optionally, the possibility remains to filter out combinations that correspond with excessive analysis times.

A program utilising this algorithm was written using the Visual Basic platform of Microsoft Excel. This program performs all optimization calculations and automatic searching of optimal stationary phase combinations. The software is available, free of charge, from the Pfizer Analytical Research Centre, Ghent University, Belgium and from Bischoff Chromatography.

The algorithm for prediction of retention times for linear gradient conditions was first evaluated with a mixture of 8 test compounds: acetophenone, aniline, caffeine, phenol, pyridine, benzene, propylparaben and toluene. The mobile phase consisted of ACN and water. A dwell time of 60 s was measured on the HPLC system. The algorithm was tested in the first instance on a 10 cm column with the C18 SH 2 stationary phase. To obtain the experimental parameters of Eq. 4.1 (*a, b* and *c*) for each compound on each stationary phase, a number of preliminary isocratic measurements, each with a different φ, is required. The difference between 3 (at 90%, 50% and 10% v/v ACN) and 8 or 9 (at (90%), 80%, 70%, 60%, 50%, 40%, 30%, 20% and 10% v/v ACN) preliminary isocratic measurements was investigated. The non-linear fits with Eq. 4.1 for the plots representing *ln(k)* versus φ for phenol for 3 and 9 preliminary measurements are shown in Figure 4.2. Comparison of the retention times, predicted according to Eq. 4.1, based on 3 and 8 or 9 preliminary measurements, of the test compounds is shown in Table 4.1, together with the experimental retention times for different linear gradient profiles. As can be noted, the 3 solvent composition experiment provided nearly as good data as the 9 solvent composition experiment. The requirement hereby is that the 3 solvent composition experiments are performed at equidistant isocratic levels over the range of φ.

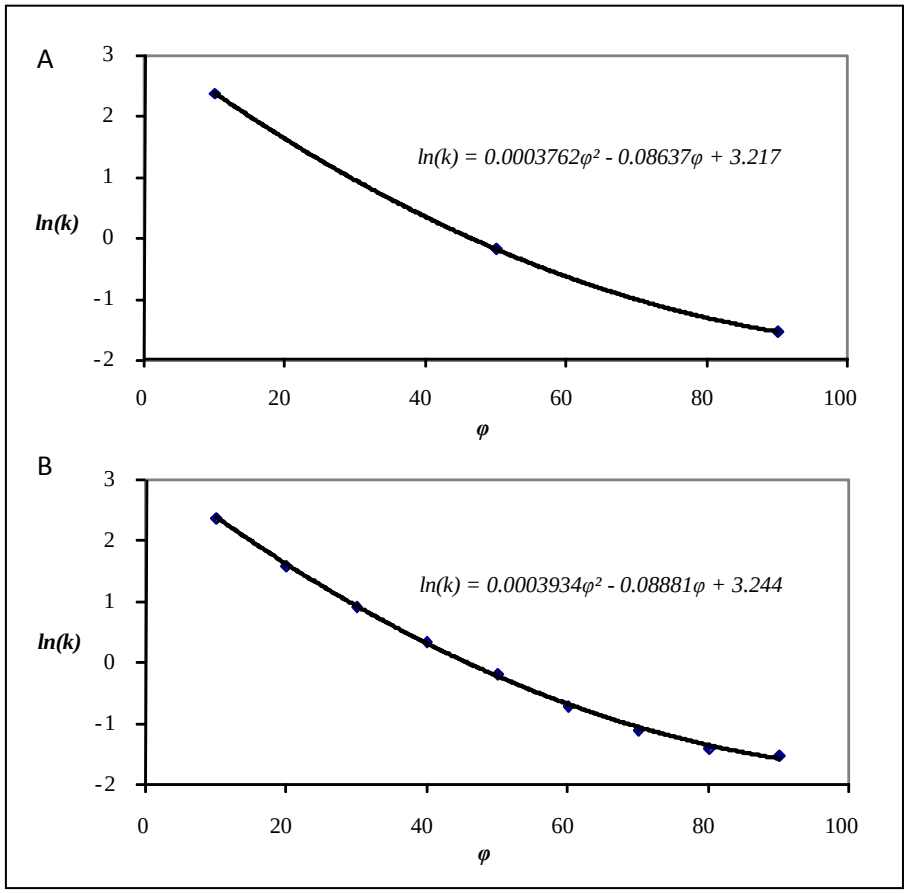

Fig. 4.2 Acquisition of ln(k) = aφ² + bφ + c, based on 3 (A) and 9 (B) preliminary isocratic measurements for phenol a on a 10 cm C18 SH 2 column.

Table 4.1 Comparison of experimental and predicted retention times for 8 test compounds, based on 3 and 8 or 9 preliminary isocratic measurements, using multiple gradient profiles on a 10 cm C18 SH 2 column.

H_2O/ACN: 90/10 → 10/90 in 15 min flow: 1mL min^{-1} 10 cm C18 SH 2					
compounds	retention time predictions (min.) based on 9 prel. measurements	retention time predictions (min.) based on 3 prel. measurements	experimental retention times (min.)	deviation (min.) between exp. and pred. based on 9 prel. measurements	deviation (min.) between exp. and pred. based on 3 prel. measurements
Acetophenone	6.47	6.52	6.51	0.04	-0.01
Aniline	3.92	3.93	3.93	0.01	0.00
Caffeine	2.70	2.80	2.80	0.10	0.00
Phenol	4.32	4.35	4.47	0.15	0.12
Pyridine	2.93	3.00	3.03	0.10	0.03
H_2O/ACN: 80/20 → 10/90 in 15 min flow: 1mL min^{-1} 10 cm C18 SH 2					
compounds	retention time predictions (min.) based on 8 prel. measurements	retention time predictions (min.) based on 3 prel. measurements	experimental retention times (min.)	deviation (min.) between exp. and pred. based on 8 prel. measurements	deviation (min.) between exp. and pred. based on 3 prel. measurements
Benzene	6.93	6.95	7.10	0.17	0.15
Propylparaben	6.55	6.62	6.71	0.16	0.09
Toluene	8.62	8.68	8.86	0.24	0.18
H_2O/ACN: 90/10 → 10/90 in 30 min flow: 1mL min^{-1} 10 cm C18 SH 2					
compounds	retention time predictions (min.) based on 9 prel. measurements	retention time predictions (min.) based on 3 prel. measurements	experimental retention times (min.)	deviation (min.) between exp. and pred. based on 9 prel. measurements	deviation (min.) between exp. and pred. based on 3 prel. measurements
Acetophenone	8.77	8.88	8.77	0.00	-0.11
Aniline	4.38	4.38	4.31	-0.07	-0.07
Caffeine	3.03	3.03	3.14	0.11	0.11
Phenol	5.08	5.10	5.24	0.16	0.14
Pyridine	3.13	3.22	3.22	0.09	0.00
H_2O/ACN: 80/20 → 10/90 in 30 min flow: 1mL min^{-1} 10 cm C18 SH 2					
compounds	retention time predictions (min.) based on 8 prel. measurements	retention time predictions (min.) based on 3 prel. measurements	experimental retention times (min.)	deviation (min.) between exp. and pred. based on 8 prel. measurements	deviation (min.) between exp. and pred. based on 3 prel. measurements
Benzene	9.13	9.07	9.24	0.11	0.17
Propylparaben	9.15	9.30	9.24	0.09	-0.06
Toluene	12.42	12.50	12.80	0.38	0.30

For the highly retained compounds benzene, propylparaben and toluene, only the isocratic measurements at 20% v/v of ACN and higher were considered because the measurement at 10% v/v of ACN resulted in retention times above 1 hour. This led, however, to little differences between the predicted retention times.

The accuracy of the prediction of the retention times of the test compounds was further evaluated for different gradient profiles with different slopes in 30 min. The predicted retention times and the experimental retention times can be seen in Table 4.2 and can best be compared and evaluated by means of the retention time deviations.

In a next step, columns with other stationary phases were used to further evaluate the prediction algorithm. Predictions for a 10 cm C18 EPS2 stationary phase and a 10 cm C30 stationary phase are, as an example, shown in Table 4.3. Deviations ranged from 0.01 to 0.31 min. The *a*, *b* and *c* terms of the 8 test compounds are shown in Table 4.4.

Subsequently, the prediction algorithm was tested on a randomly chosen column segment combination comprised of 5 cm C18 SH2 stationary phase at the column inlet, followed by 5 cm C18 EPS 2 and ended by 5 cm C30 at the column outlet. The linear gradient profile, predictions and experimental retention times are shown in Table 4.5, together with the noted deviations. In Figure 4.3 an appended simulation of the chromatogram with the predicted retention times is shown in addition to the experimental chromatogram, graphically demonstrating the validity of the approach.

Table 4.2. Evaluation and comparison of predicted and experimental retention times for 8 test compounds for multiple linear gradient profiles on a 10 cm C18 SH 2 column.

H_2O/ACN: 90/10 → 50/50 in 30 min flow: 1mL min^{-1} 10 cm C18 SH 2			
compounds	retention time predictions (min.) based on 5 prel. measurements	experimental retention times (min.)	deviation (min.) between exp. and pred. based on 5 prel. measurements
Acetophenone	11.53	11.43	-0.10
Aniline	4.73	4.68	-0.05
Caffeine	3.33	3.37	0.04
Phenol	5.73	5.90	0.17
Pyridine	3.27	3.38	0.11
H_2O/ACN: 80/20 → 50/50 in 30 min flow: 1ml min^{-1} 10cm C18 SH 2			
compounds	retention time predictions (min.) based on 4 prel. measurements	experimental retention times (min.)	deviation (min.) between exp. and pred. based on 4 prel. measurements
Benzene	12.05	12.04	-0.01
Prop. paraben	13.35	13.64	0.29
Toluene	18.75	19.18	0.43
H_2O/ACN: 50/50 → 10/90 in 30 min flow: 1ml min^{-1} 10cm C18 SH 2			
compounds	retention time predictions (min.) based on 5 prel. measurements	experimental retention times (min.)	deviation (min.) between exp. and pred. based on 5 prel. measurements
Acetophenone	1.38	1.43	0.05
Aniline	0.98	0.99	0.01
Caffeine	0.58	0.52	-0.06
Phenol	0.93	0.95	0.02
Pyridine	0.85	0.83	-0.02
Benzene	2.43	2.53	0.10
Prop. paraben	1.58	1.70	0.12
Toluene	3.53	3.74	0.21

Table 4.3 Evaluation and comparison of predicted and experimental retention times for 8 test compounds on a 10 cm C18 EPS 2 column and a 10 cm C30 column.

H_2O/ACN: 90/10 → 10/90 in 30 min flow: 1mL min^{-1} 10 cm C18 EPS2			
compounds	retention time predictions (min.) based on 9 prel. measurements	experimental retention times (min.)	deviation (min.) between exp. and pred. based on 9 prel. measurements
Acetophenone	7.47	7.53	0.06
Aniline	3.52	3.49	-0.03
Caffeine	2.73	2.58	-0.15
Phenol	6.23	6.30	0.07
Pyridine	1.97	1.96	-0.01
H_2O/ACN: 80/20 → 10/90 in 30 min flow: 1mL min^{-1} 10 cm C18 EPS2			
compounds	retention time predictions (min.) based on 8 prel. measurements	experimental retention times (min.)	deviation (min.) between exp. and pred. based on 8 prel. measurements
Benzene	8.13	8.21	0.08
Prop. paraben	11.60	11.34	-0.26
Toluene	11.25	11.56	0.31
H_2O/ACN: 80/20 → 10/90 in 30 min flow: 1mL min^{-1} 10 cm C30			
compounds	retention time predictions (min.) based on 8 prel. measurements	experimental retention times (min.)	deviation (min.) between exp. and pred. based on 8 prel. measurements
Acetophenone	4.20	4.19	-0.01
Aniline	2.12	2.11	-0.01
Caffeine	1.47	1.49	0.02
Phenol	2.18	2.13	-0.05
Pyridine	1.80	1.79	-0.01
Benzene	5.57	5.50	-0.07
Prop. paraben	7.57	7.47	-0.10
Toluene	8.46	8.61	0.15

Table 4.4 Overview of the a, b and c terms of the 8 test compounds on the C18 SH2, C18 EPS2 and C30 stationary phases.

	***a, b* and *c* terms of the compound based on 9 (or 8*) preliminary measurements on the C18 SH2 stationary phase**		
compounds	***a* (x 10^4)**	***b* (x 10^2)**	***c***
Acetophenone	5.16	-10.7	4.63
Aniline	3.03	-7.25	2.80
Caffeine	31.1	-28.1	4.26
Phenol	3.93	-8.88	3.24
Pyridine	6.76	-9.34	2.47
Benzene*	3.24	-9.11	5.15
Propylparaben*	8.36	-16.2	6.86
Toluene*	4.34	-11.2	6.45
	***a, b* and *c* terms of the compound based on 9 (or 8*) preliminary measurements on the C18 EPS2 stationary phase**		
compounds	***a* (x 10^4)**	***b* (x 10^2)**	***c***
Acetophenone	4.30	-9.44	4.05
Aniline	2.42	-6.22	2.33
Caffeine	22.9	-27.0	4.00
Phenol	3.79	-8.21	3.48
Pyridine	6.68	-9.14	1.72
Benzene*	2.79	-8.35	4.69
Propylparaben*	8.54	-15.9	7.27
Toluene*	4.18	-10.6	5.98
	***a, b* and *c* terms of the compound based on 9 (or 8*) preliminary measurements on the C30 stationary phase**		
compounds	***a* (x 10^4)**	***b* (x 10^2)**	***c***
Acetophenone	3.91	-9.80	3.78
Aniline	2.23	-7.12	2.27
Caffeine	13.7	-16.8	3.12
Phenol	2.18	-8.17	2.52
Pyridine	6.21	-8.87	2.21
Benzene*	1.99	-8.04	3.92
Propylparaben*	7.05	-15.4	6.10
Toluene*	3.43	-10.4	5.26

Table 4.5 Evaluation and comparison of predicted and experimental retention times for 8 test compounds on a combined column consisting of 5 cm C18 SH2 at the beginning, followed by a connection of 5 cm C18 EPS 2 and ending with a connection of 5 cm C30.

H_2O/ACN: 80/20 → 10/90 in 30 min **flow: 1mL min^{-1} 5 cm C18 SH2 + 5 cm C18 EPS2 + 5 cm C30**			
compounds	retention time predictions (min.) based on 8 prel. measurements	experimental retention times (min.)	deviation (min.) between exp. and pred. based on 8 prel. measurements
1. Caffeine	1.73	1.72	-0.01
2. Pyridine	2.43	2.45	0.02
3. Aniline	3.53	3.51	-0.02
4. Phenol	4.48	4.60	0.12
5. Acetophenone	6.80	6.72	-0.08
6. Benzene	9.98	10.12	0.14
7. Propylparaben	11.58	11.62	0.04
8. Toluene	13.17	13.59	0.42

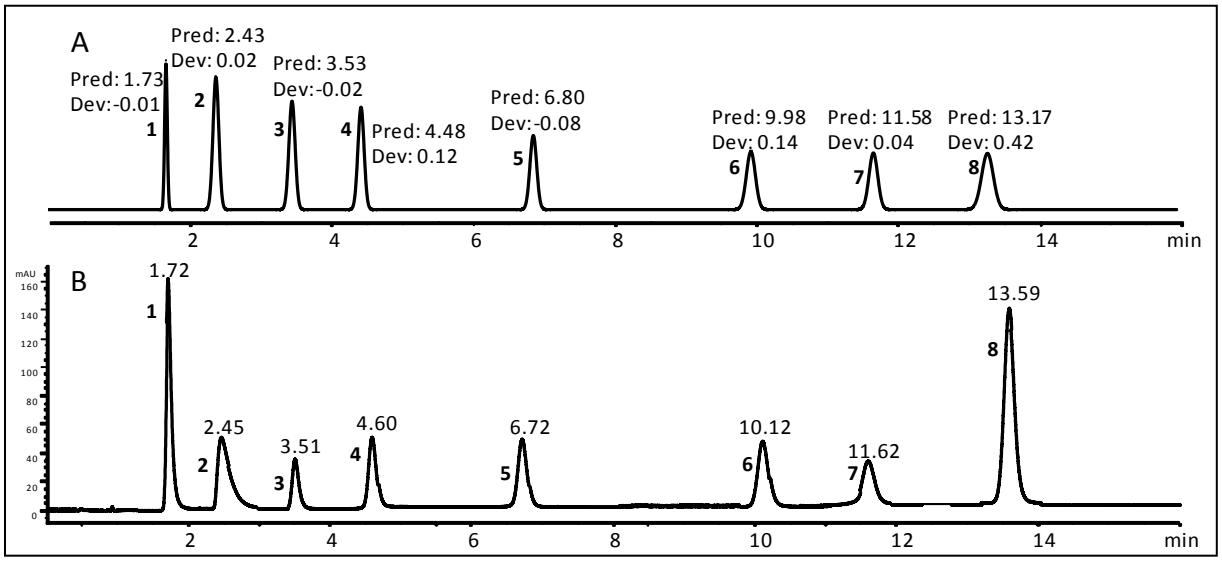

Fig. 4.3 Simulation (A) and experimental (B) chromatogram for the separation of 8 test compounds on 5 cm C18 SH 2 (at column inlet), followed by 5 cm C18 EPS 2 and 5 cm C30 (at column outlet). Peak numbering: see Table 4.5.

To further probe the features of the prediction algorithm for linear gradient SOSLC, a steroid mixture was prepared and analysed on the 5 available POPLC stationary phases at 20%, 30%, 40%, 50%, 60%, 70%, 80% and 90% v/v ACN in order to obtain a fit to Eq. 4.1 for all compounds on the 5 different stationary phases. All prediction calculations were performed for a commonly used linear gradient profile, starting from min 0 at 20% ACN (v/v) and going to 90% (v/v) of ACN in 30 min. As 5 different stationary phases were used and as all lengths up to 25 cm were considered (in segments of 1 cm) and stationary phase order had to be taken into consideration, the retention times of all steroids were predicted for 17.100.600 different combinations. Note that this vastly outnumbers the isocratic predictions where "only" 142.505 combinations were considered. The ranking of the combinations was based on the retention time difference of the critical peak pair of the combinations. An optimal combination consisting of 9 cm C18 SH2 at the column inlet, followed by 1 cm C18 EPS2 and ending with 8 cm C30 was determined. This resulted in a total column length of 18 cm and corresponded to the highest value for the retention time difference of the critical peak pair of all combinations, whilst enabling analysis within a stipulated maximum time limit of 30 min. A simulation of the chromatogram with predicted retention times together with the experimental chromatogram are shown in Figure 4.4. The retention time deviations are shown in Table 4.6. The *a*, *b* and *c* terms of the steroids on the C18 SH2, C18 EPS2 and C30 stationary phases are shown in Table 4.7.

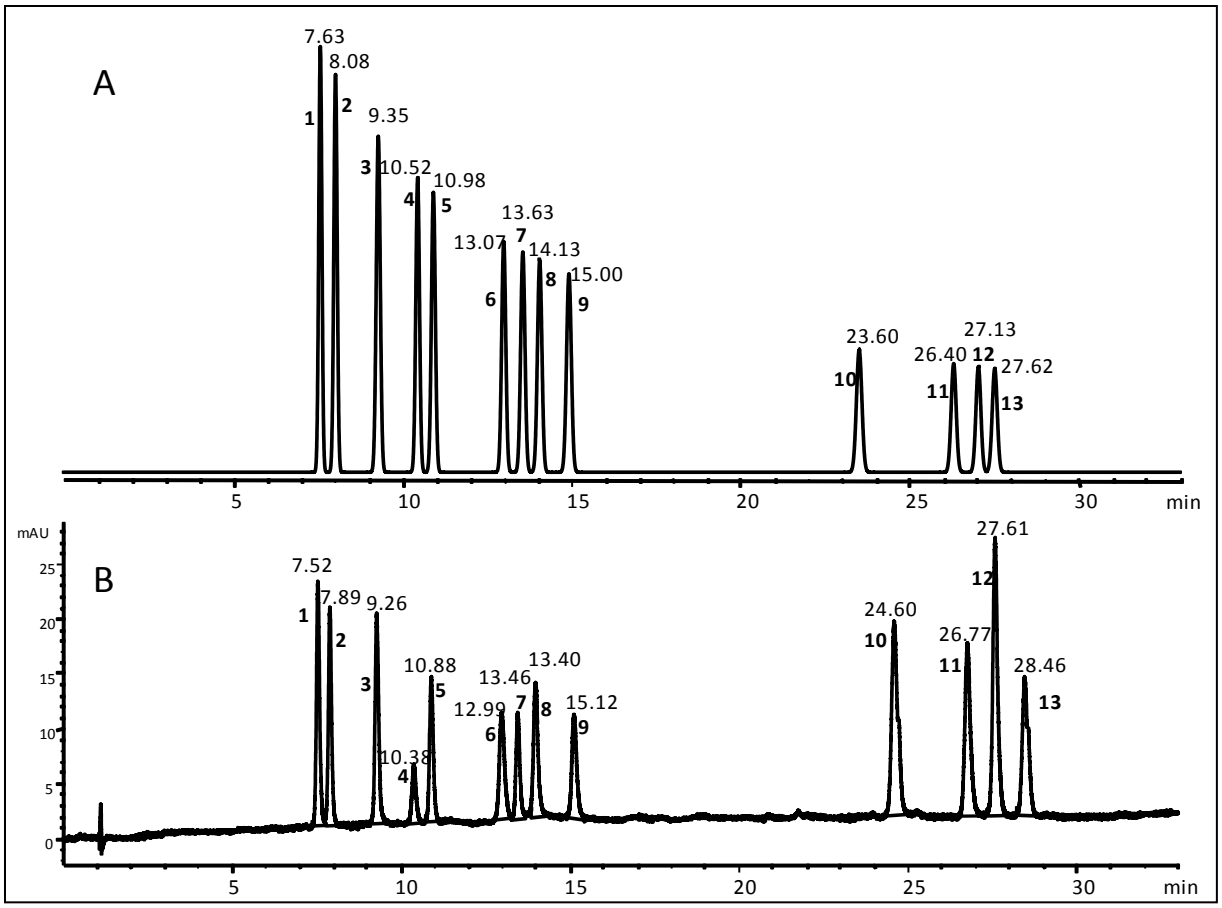

Fig. 4.4 Simulation (A) and experimental (B) chromatogram for the separation of a steroid mixture on 9 cm C18 SH 2 + 1 cm C18 EPS 2 + 8 cm C30 (total 18 cm), for optimal separation within 30 min with a fixed gradient profile: H_2O/ACN: 80/20 to 10/90 in 30 minutes, starting from min 0. For identification of peak numbers, please refer to Table 4.6.

Table 4.6 Evaluation and overview of predicted and experimental retention times for compounds of a steroid mixture using an optimized column combination, 9 cm C18 SH 2 + 1 cm C18 EPS 2 + 8 cm C30 (total 18 cm), for optimal separation within 30 min with a fixed gradient profile: H_2O/ACN: 80/20 to 10/90 in 30 min, starting from min 0.

H_2O/ACN: 80/20 → 10/90 in 30 min flow: 1mL min^{-1} Optimized column combination: 9 cm C18 SH2 + 1 cm C18 EPS2 + 8 cm C30			
compounds	retention time predictions (min.) based on 8 prel. measurements	experimental retention times (min.)	deviation (min.) between exp. and pred.
1. Prednisolone	7.63	7.52	-0.09
2. Cortisone	8.08	7.89	-0.19
3. Me-Prednisolone	9.35	9.26	-0.09
4. Triamcinolone acetonide	10.52	10.38	-0.14
5. Cortexolone	10.98	10.88	-0.10
6. Me-Boldenone	13.07	12.99	-0.08
7. Oestradiol	13.63	13.46	-0.17
8. Testosterone	14.13	14.00	-0.13
9. Me-Testosterone	15.00	15.12	0.12
10. Testosteron propionate	23.60	24.60	1.00
11. Progesterone	26.40	26.77	0.37
12. Testosterone phenyl propionate	27.13	27.61	0.48
13. Nortestosterone	27.62	28.46	0.84

Table 4.7 Overview of the a, b and c terms of the steroids on the C18 SH2, C18 EPS2 and C30 stationary phases.

	***a, b* and *c* terms of the compound based 8 preliminary measurements on the C18 SH2 stationary phase**		
compounds	***a* (x 10^4)**	***b* (x 10^1)**	***c***
Prednisolone	16.5	-2.44	7.23
Cortisone	14.7	-2.27	7.10
Me-Prednisolone	16.3	-2.48	8.04
Triamcinolone acetonide	13.3	-2.20	7.93
Cortexolone	13.5	-2.16	7.84
Me-Boldenone	12.4	-2.03	8.15
Oestradiol	12.0	-2.10	8.57
Testosterone	12.3	-2.04	8.45
Me-Testosterone	12.5	-2.08	8.94
Testosteron propionate	10.4	-2.11	11.7
Progesterone	12.9	-2.56	13.7
Testosterone phenyl propionate	9.41	-2.16	13.3
Nortestosterone	13.0	-2.63	14.5
	***a, b* and *c* terms of the compound based on 8 preliminary measurements on the C18 EPS2 stationary phase**		
compounds	***a* (x 10^4)**	***b* (x 10^1)**	***c***
Prednisolone	15.0	-2.16	6.67
Cortisone	13.5	-2.07	6.60
Me-Prednisolone	15.2	-2.25	7.57
Triamcinolone acetonide	13.5	-2.16	7.87
Cortexolone	13.5	-2.11	7.81
Me-Boldenone	12.7	-2.04	8.07
Oestradiol	12.8	-2.14	9.48
Testosterone	12.6	-2.05	8.45
Me-Testosterone	12.8	-2.11	8.87
Testosteron propionate	13.7	-2.52	12.5
Progesterone	13.7	-2.65	14.0
Testosterone phenyl propionate	13.6	-2.73	14.7
Nortestosterone	12.9	-2.60	14.0
	***a, b* and *c* terms of the compound based on 8 preliminary measurements on the C30 stationary phase**		
compounds	***a* (x 10^4)**	***b* (x 10^1)**	***c***
Prednisolone	15.8	-2.35	6.92
Cortisone	14.1	-2.23	6.83
Me-Prednisolone	16.9	-2.49	7.83
Triamcinolone acetonide	13.7	-2.29	7.84
Cortexolone	14.1	-2.26	8.00
Me-Boldenone	13.6	-2.19	8.35
Oestradiol	13.2	-2.27	8.83
Testosterone	13.8	-2.24	8.87
Me-Testosterone	14.1	-2.30	9.26
Testosteron propionate	11.5	-2.26	11.8
Progesterone	14.0	-2.71	14.3
Testosterone phenyl propionate	13.4	-2.72	17.7
Nortestosterone	13.5	-2.70	14.4

A full baseline separation was achieved by means of SOSLC under linear gradient conditions. Given the applied gradient profile, it is clear that an isocratic separation would be far from useful for analysis of this steroid mixture.

The real advantage of SOSLC is that the selectivity for a given separation can be fine-tuned by combining different stationary phases in a practical way. Selecting at random one stationary phase to achieve that goal can be problematic. In Figure 4.5, the chromatographic runs under the same conditions on 18 cm columns of each individual stationary phase are shown. While no attempt was made to optimize the separation on these columns, insufficient separation is noted for all individual phases. Mobile phase optimization or increasing the column efficiency can provide an answer, but applying linear gradient SOSLC is definitely an attractive alternative approach.

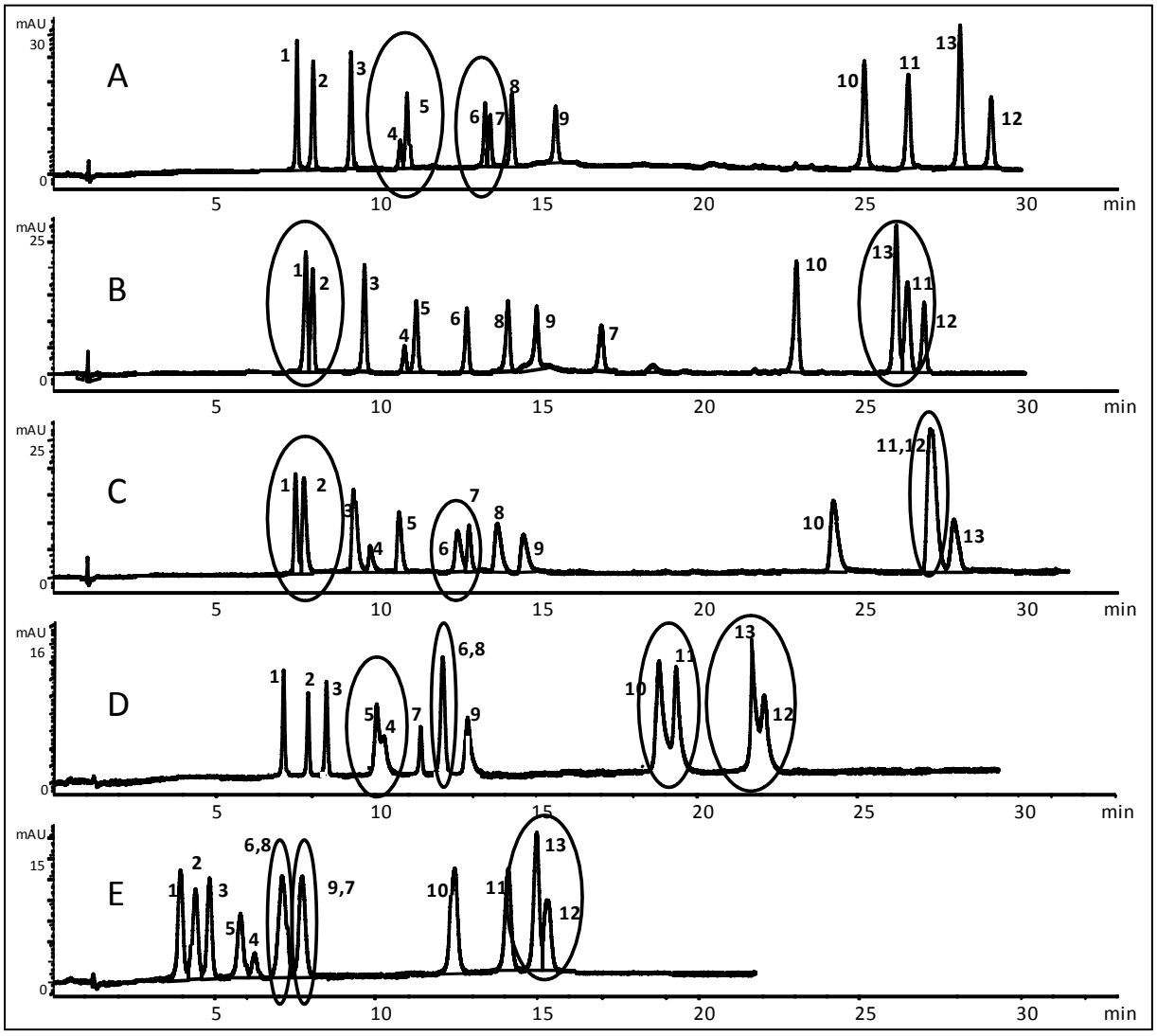

Fig. 4.5 Comparison between 18 cm columns for each different stationary phase using a fixed gradient profile: H_2O/ACN: 80/20 to 10/90 in 30 minutes, starting from minute 0. A: C18 SH 2, B: C18 EPS 2, C: C30, D: Phenyl, E: CN. For identification of the peak numbers, refer to Table 4.6.

4.4 Conclusions

The concept of SOSLC has been extended to linear gradient conditions whereby retention time predictions of all compounds of a mixture are calculated with acceptable accuracy. A minimum of 3 preliminary measurements on each individual stationary phase remains, however, mandatory in order to obtain retention profiles of compounds as a function of the organic modifier concentration. Due to the discontinue character of the mathematical algorithm for predicting retention times, other types then linear gradients can be considered for SOSLC as well. In this context, multi-linear gradients are described in Chapter 4 where emphasis has been set on green chromatography.

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Chapter 5: Selectivity optimization in green chromatography by gradient stationary phase optimized selectivity chromatography

Summary

Stationary phase optimized selectivity liquid chromatography (SOSLC) is a promising technique to optimize the selectivity of a given separation by using a combination of different stationary phases. Previous chapters have shown that SOSLC offers excellent possibilities for method development, especially after the modification towards linear gradient SOSLC. The content of this chapter aimed at developing and extending the SOSLC approach towards selectivity optimization and method development for green chromatography. Contrary to current LC practices, a green mobile phase (water/ethanol/formic acid) is hereby preselected and the composition of the stationary phase is optimized under a given gradient profile to obtain baseline resolution of all target solutes in the shortest possible analysis time. With the algorithm adapted to the high viscosity property of ethanol, the principle is illustrated with a fast, full baseline resolution for a randomly selected mixture composed of sulphonamides, xanthine alkaloids and steroids.

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5.1 Introduction

Selectivity optimization is arguably the most important aspect of HPLC method development as it influences the resolution of a separation in a much more significant way compared to the retention factor and the column efficiency, as has been described in Chapter 2. As described before, the optimization process is, in most cases, performed partially on experimental evidence and partially on personal experience. This combination of somewhat arbitrary choices and of incomplete experimental investigations often results in the selection of conditions which do not necessarily correspond to the best possible selectivity [1–5].

Recently, with the shortage and high price of acetonitrile, the concept of green chromatography has regained attention [6–10]. The aim is hereby to minimize or eliminate the usage of environmentally hazardous organic solvents. In green chromatography, conventional organic modifiers in reversed-phase LC (RP-LC) such as acetonitrile (moderate toxicity [11–13]) or additives such as trifluoroacetic acid which is highly ecotoxic and is slow to biodegrade [14], are substituted with environmentally friendly alternatives such as ethanol and formic acid. Ethanol is categorised as a 'green' solvent due to its low toxicity, potential synthesis from renewable feedstocks but most importantly because of its low lifecycle impact on the environment – that is its low ecological impact from synthesis through use and ease of recycling or disposal [11,13,15]. Similarly, formic acid is classed as a green solvent principally due to its rapid biodegradation to benign by-products, i.e. carbon dioxide and water [14]. The development of a green chromatographic method therefore introduces the conceptual change to first choose the (green) mobile phase constituents followed by optimization of the remaining parameters which mainly comprises the choice of stationary phase.

With SOSLC [16–20] the mobile phase is preselected after which the stationary phase is optimized. This approach is therefore ideally suited for green chromatography application. As described in Chapters 3 and 4, a major limitation of the PRISMA model is that it is limited to isocratic analysis. To overcome involved limitations, a multiple step gradient method was therefore developed [21]. In the multiple step gradient optimizations described in Chapter 3, the retention time prediction was still based on isocratic measurements, which leads to certain deviations from the actual step-gradient analysis. Secondly, the order of segments was not taken into consideration in this approach, which also influences the retention times to some extent in gradient analysis. To solve these problems, a linear gradient algorithm for SOSLC was developed and described in Chapter 4 [22]. Retention models of the compounds on the stationary phases are thereby first built as a function of the organic modifier concentration. The gradient elution is then considered as a sequence of small isocratic

stages, for which the migrated distance and time of each analyte band can be calculated. The accumulated migration time of all the small isocratic stages is finally used as the predicted retention time in the gradient elution. The algorithm can as well be used in the isocratic, step-wise and linear gradient run mode.

In this contribution, the features of gradient SOSLC are demonstrated for green chromatography. Green mobile phase components and a fixed gradient are thereby pre-selected and the optimal column compositions (and order) are predicted, from a set of 8,037,725 unique column combinations, leading to baseline separation of the analytes. The concept is demonstrated with a mixture of 14 pharmaceutical compounds. In-house MS windows compatible software was therefore developed in order to support mathematical calculations and predictions [23].

5.2 Experimental

5.2.1 Chemicals and reagents

All chemicals and sample solutes were obtained from Sigma–Aldrich (Bornem, Belgium) except ethanol and formic acid which originated from Biosolve (Valkenswaard, The Netherlands). All stock solutions were prepared in acetonitrile. The mixture consisted of 14 pharmaceutical compounds and the individual pharmaceutical samples were prepared by diluting the stock solutions with water to a final concentration (Table 5.1). All the samples included uracil as unretained marker at a concentration of 5 µg mL^{-1}.

Table 5.1 The numbering and concentration of 14 compounds in the sample mixture.

Numbering	Compound name	Concentration in the mixture sample (μg mL^{-1})
1	Theobromine	800
2	Theophylline	50
3	Caffeine	100
4	Sulfadiazine	200
5	Sulfamerazine	50
6	Sulfamethazine	30
7	Sulfamethizole	30
8	Sulfamethoxazole	75
9	Estriol	1000
10	Sulfadimethoxine	75
11	Sulfaquinoxaline	200
12	Prednisone	75
13	Prednisolone	75
14	Cortisone	150

5.2.2 Instrumentation

All experiments were performed on an Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) and with a POPLC® Basic Kit 250-5 (Bischoff Chromatography, Leonberg, Germany). The kit consists of five stationary phases: ProntoSIL C18 EPS 2, ProntoSIL C18 SH 2, ProntoSIL C30, ProntoSIL CN 2 and ProntoSIL Phenyl 2. Each stationary phase has a set of column segments with lengths of 10, 20, 40 (2×), 60 and 80mm, which ensures the possibility of a segment combination of a single stationary phase from 1 to 25 cm. The column segments of all the five stationary phases have a particle size of 5 μm and the internal diameter is 3 mm. Chemstation software (Agilent Technologies) was used for data collection and peak integration. For stationary phase optimization, POPLC® Optimizer v 1.04.03 (Bischoff Chromatography) was used under isocratic conditions and the software developed by our group was used for the predictions under gradient conditions [23].

5.2.3 Chromatographic conditions

After preliminary tests, the column temperature was set at 50 °C and the flow rate at 0.5mL min^{-1}. The wavelength of the VWD detector was set at 254 nm and the injected volumes were 1 µL. The mobile phases were composed of water with 0.1% formic acid and ethanol. For isocratic optimization, the mobile phase composition was 85% (v/v) water and 15% (v/v) ethanol. For gradient optimization, the basic measurements were performed at 8 isocratic levels, i.e. 10%, 15%, 20%, 25%, 30%, 35%, 40% and 45% (v/v) ethanol and the column length was 10 cm for all the five stationary phases. The mobile phase was post-mixed by a binary pump. A linear gradient condition was then employed for gradient optimization from 10% to 50% (v/v) ethanol in 30 min on the optimized segment combination. A detailed list of the experiments is included in Table 5.2.

Table 5.2 List of experiments. Common conditions: the column temperature was set at 50 °C and the flow rate at 0.5 mL min^{-1}. The wavelength of the VWD detector was set at 254 nm and the injected volumes were 1 µL. The mobile phases were composed of water with 0.1% formic acid (solvent A) and ethanol (solvent B).

Numbering	Type	Mobile phase	Stationary phase	Sample
1	basic measurements	isocratic, 90% A + 10% B	10 cm single segment (five stationary phases respectively)	single compound (uracil and 14 compounds respectively)
2	basic measurements	isocratic, 85% A + 15% B	10 cm single segment (five stationary phases respectively)	single compound (uracil and 14 compounds respectively)
3	basic measurements	isocratic, 80% A + 20% B	10 cm single segment (five stationary phases respectively)	single compound (uracil and 14 compounds respectively)
4	basic measurements	isocratic, 75% A + 25% B	10 cm single segment (five stationary phases respectively)	single compound (uracil and 14 compounds respectively)
5	basic measurements	isocratic, 70% A + 30% B	10 cm single segment (five stationary phases respectively)	single compound (uracil and 14 compounds respectively)
6	basic measurements	isocratic, 65% A + 35% B	10 cm single segment (five stationary phases respectively)	single compound (uracil and 14 compounds respectively)
7	basic measurements	isocratic, 60% A + 40% B	10 cm single segment (five stationary phases respectively)	single compound (uracil and 14 compounds respectively)
8	basic measurements	isocratic, 55% A + 45% B	10 cm single segment (five stationary phases respectively)	single compound (uracil and 14 compounds respectively)
9	isocratic optimization	isocratic, 85% A + 15% B	1 cm C30 + 4 cm C18 + 12 cm C18EPS	mixture sample
10	gradient optimization, Rank 1	gradient from 10% B to 50% B in 30 minutes	6 cm C18 + 12 cm C18EPS	mixture sample
11	gradient optimization, Rank 20	gradient from 10% B to 50% B in 30 minutes	5 cm C18 + 12 cm C18EPS + 1 cm CN	mixture sample
12	gradient optimization, Rank 50	gradient from 10% B to 50% B in 30 minutes	8 cm C18EPS + 5 cm C30 + 5 cm Phenyl	mixture sample

5.3 Theory and algorithm

5.3.1 Isocratic optimization

The migration time of a certain compound on a given stationary phase segment can be described as the upper limit of the integral in Eq. 5.1. The retention time on the combined column can therefore be calculated by Eq. 5.2:

$$l_i = \int_{t=0}^{t_{t,i}} v_i dt = \int_{t=0}^{t_{r,i}} \frac{u}{(1+k_i)} dt \qquad \text{(Eq. 5.1)}$$

$$t_r = \sum_{i=1}^{5} t_{ri} + t_{0,EC} \qquad \text{(Eq. 5.2)}$$

where l_i is the length of the ith segment, v_i is the linear velocity of an analyte band, k_i is the retention factor of the analyte on the ith segment and u is the linear velocity of the mobile phase determined by the elution time of an unretained marker (uracil). $T_{0,EC}$ is the extra-column void time measured by the elution time of uracil through the system whereby the column is replaced by a union. $t_{r,i}$ is the retention time on the ith segment and t_r is the retention time on the combined column including the system void time. Under isocratic conditions, the retention factor k_i is a constant and Eq. 5.1 can be rewritten to Eqs. 5.3 and 5.4.

$$l_i = \frac{u}{(1+k_i)} \int_{t=0}^{t_{r,i}} dt = \frac{u}{(1+k_i)} t_{r,i} \qquad \text{(Eq. 5.3)}$$

$$t_{r,i} = \frac{(1+k_i)}{u} l_i \qquad \text{(Eq. 5.4)}$$

Because the total length of the combined column L is the sum of the lengths of segments l_i (Eq. 5.5), Eq. 5.6 can be obtained by replacing $t_{r,i}$ in Eq. 5.2 with Eq. 5.4. Under isocratic conditions, the retention factor k_i is a constant and Eq. 5.1 can be rewritten to Eqs. 5.3 and 5.4.

$$L = \sum_{i=1}^{5} l_i \quad \text{(Eq. 5.5)}$$

$$t_r = \sum_{i=1}^{5} \frac{(1+k_i)}{u} l_i + t_{0,EC} = \frac{L}{u} + \frac{1}{u}\sum_{i=1}^{5} k_i l_i + t_{0,EC} \quad \text{(Eq. 5.6)}$$

Assuming that the combined column has an experimental retention factor k_c, it can be defined and rewritten as Eq. 5.7. Eq. 5.8 is obtained by combining Eq. 5.6 and Eq. 5.7.

$$t_r = \frac{L}{u}(1 + k_c) + t_{0,EC} = \frac{L}{u} + \frac{L}{u}k_c + t_{0,EC} \quad \text{(Eq. 5.7)}$$

$$k_c = \frac{\sum_{i=1}^{5} k_i l_i}{L} \quad \text{(Eq. 5.8)}$$

Equation 5.8 corresponds to the PRISMA model valid for isocratic conditions [17].

7.3.2 Gradient optimization

Under gradient conditions, however, the retention factor k is no longer a constant. For a single stationary phase, the retention factor k_i at a given time point is mainly influenced by the composition of the mobile phase which is around the position of the analyte band. To investigate the relationship between the retention factor and the composition of mobile phase, the classical LSS retention model [24] assumes a linear relationship between the logarithm of the retention factor, ln(k) and the volume fraction of the organic modifier φ in the mobile phase, as shown in Eq. 2.16 where b and c are the coefficients which can be obtained by a linear regression.

The LSS model is widely used and has adequate accuracy in retention time prediction under gradient conditions, especially when a relative small range of φ is involved [25,26]. However, the relationship between ln(k) and φ is no longer linear if the whole range of φ is investigated as shown in Figure 5.1.

In the work presented in this Chapter, the second order polynomial expression developed by Schoenmakers et al. [27,28] is therefore used to obtain an improved relationship between (k) and φ (Eq. 2.15) where a, b and c are the coefficients obtained from the quadratic regression model based on the basic isocratic measurements with at least 3 different levels of the volume fraction of organic modifier. In practice, the number as well as the set of φ values can be different for each compound. For the sake of time saving, the basic measurements for the compounds with large retention will be investigated with relative large φ values. On the other hand, relative small φ values are set in the basic measurements for the compounds with small retention, so that more accurate values of the retention factor can be obtained. In this chapter, basic measurements of at least 5 isocratic levels are investigated for each compound for the enhancement of the regression model's reliability (Fig. 5.1).

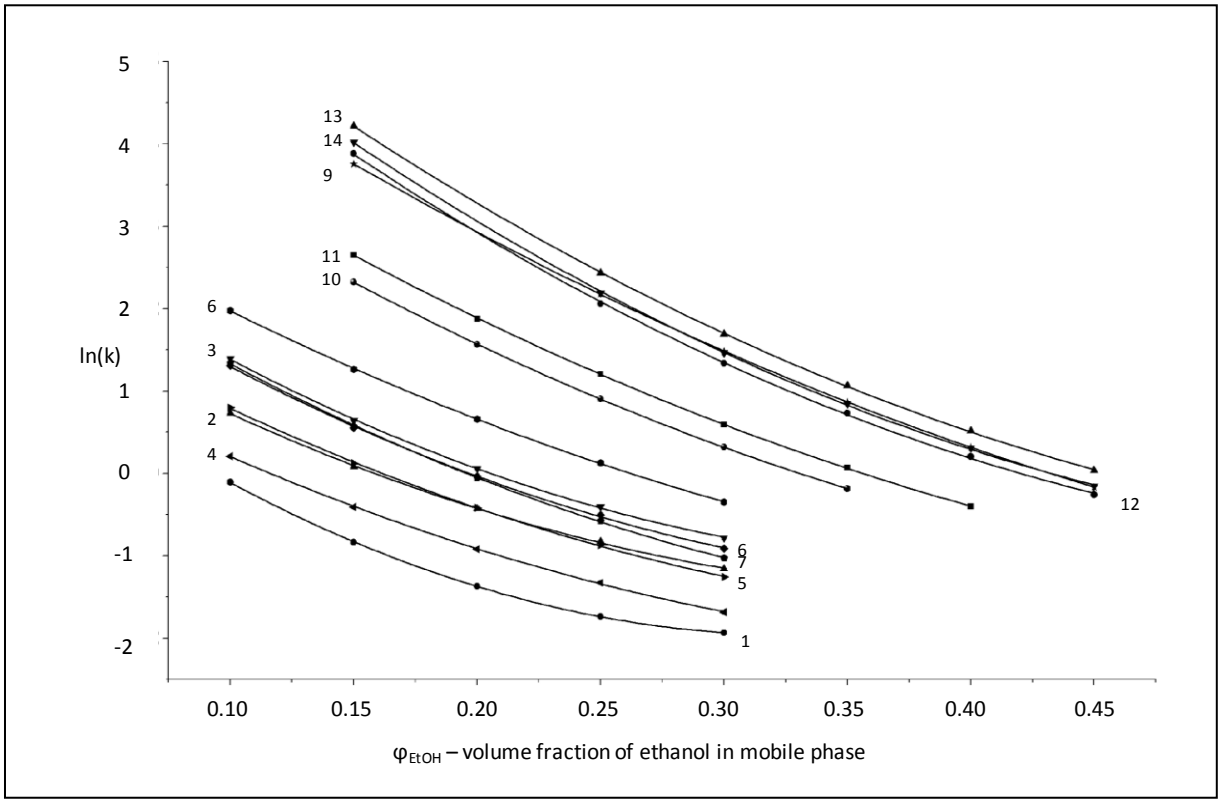

Fig. 5.1 Quadratic regression of ln(k) vs. φ_{EtOH} on 10 cm C18 SH2column. At least 5 isocratic levels are used in the basic measurements for each compound. For compound identification, please refer to Table 5.1.

In gradient conditions, however, the instant volume fraction of modifier is dependent on both the time elapsed and the distance migrated by the analyte band. A solution to predict gradient retention was shown by Schoenmakers et al. for single phases [29]. Neue and Kuss recently proposed alternatives to Eq. 2.15 to predict retention times more accurately, especially when predictions are made making use of extrapolations outside the range of measurements [30,31]. However, in this chapter Eq. 2.15 was used for the calculations of k as broad ranges of φ were used in the initial measurements. Because in the gradient SOSLC procedure the calculation of the retention time of each analyte is required for all 8,037,725 unique column combinations, and if whished for a variety of gradient profiles, a numerical solution was strongly favored for the sake of simplicity. Note that the POPLC® Basic Kit allows only 142,505 possible column combinations because, under isocratic conditions, the segment order is of no influence to the predicted retention times. This number is significantly larger when gradients are applied. Although numerical approaches are less elegant than integrated solutions they have been used quite extensively before for the prediction of retention time in chromatography [32–37].

The basic numerical integration algorithm is described as follows. Assume that t_e is the time elapsed after the analyte band enters the front of the first segment, d_m is the migration distance of the analyte band from the front of the first segment and i is the numbering of the segment where the analyte is passing by.

$$\varphi = f(t_e - \frac{d_m}{u} - t_{0,EC} - t_{dwell} \quad \text{(Eq. 5.9)}$$

$$ln(k) = a_i\varphi^2 + b_i\varphi + c_i \quad \text{(Eq. 5.10)}$$

$$d_{m,updated} = d_m + u\frac{1}{1+k}\Delta t \quad \text{(Eq. 5.11)}$$

$$t_{e,updated} = t_e + \Delta t \quad \text{(Eq. 5.12)}$$

where f(t) is the gradient function of volume fraction of organic modifier dependant on time t. Note that the function f(t) can be defined as for multi-linear gradient or any other gradient profile. If t is

less than 0, f(t) equals f(0), the φ value at the starting point of the gradient profile. t_{dwell} is the dwell time which is needed for the front of mobile phase to migrate from the solvent mixer to the inlet of the column. The coefficients a_i, b_i and c_i are the coefficients of the analyte's retention model on ith segment in the combined column. Δt, in general set to 1 s, is the constant and small time span for which the gradient elution is considered as an isocratic stage. Smaller Δt can be used if more accurate results are required despite of the longer computing time. As the iterative variable, $d_{m,updated}$ is the updated migration distance of the analyte band after the time increment of Δt in an iteration and then substitutes for d_m in the next iteration. Similarly, $t_{e,updated}$ is the updated elapsed time with an addition of Δt to te for each iteration. The iteration process of Eqs. 5.9–5.12 starts with the variables t_e and d_m set to 0. The segment numbering i will be increased by 1 if d_m reaches the next segment. Once d_m reaches the total length of the combined column, the iteration process stops and the retention time t_r will be the addition of t_e and $t_{0,EC}$.

Given a fixed gradient profile and a restriction on the maximum analysis time, the retention times of all the compounds in a mixture can be predicted for all the possible column segment combinations. For each segment combination, the retention time difference is calculated for the most critical pair of adjacent peaks and employed as the ranking score of this combination. Finally, all the possible column combinations are sorted according to the ranking scores in descending order. The segment combination at the top of the list can then be selected and employed as the optimal column. It should be noted that the retention time difference of the peaks of the critical pair instead of the selectivity factor α is employed in this work as the ranking factor. Because the resolution of a separation under a linear gradient condition is defined by the retention time difference and a nearly constant average peak width of adjacent eluted peaks, the difference of retention times is regarded here as a more suitable evaluation objective for the separation than the selectivity factor α, especially for the peaks with very small retention factors. The possibility of over-fitting is eliminated by a restriction on the maximum analysis time. A software package based on the above-mentioned algorithm and allowing the use of multi-linear gradients was developed in the laboratory for that purpose [23].

5.4 Results and discussion

Compared to acetonitrile, ethanol leads to a much larger viscosity in a binary solvent mixture with water, especially in the range of volume fractions from 30% to 50% (v/v) (Fig. 5.2) [38].

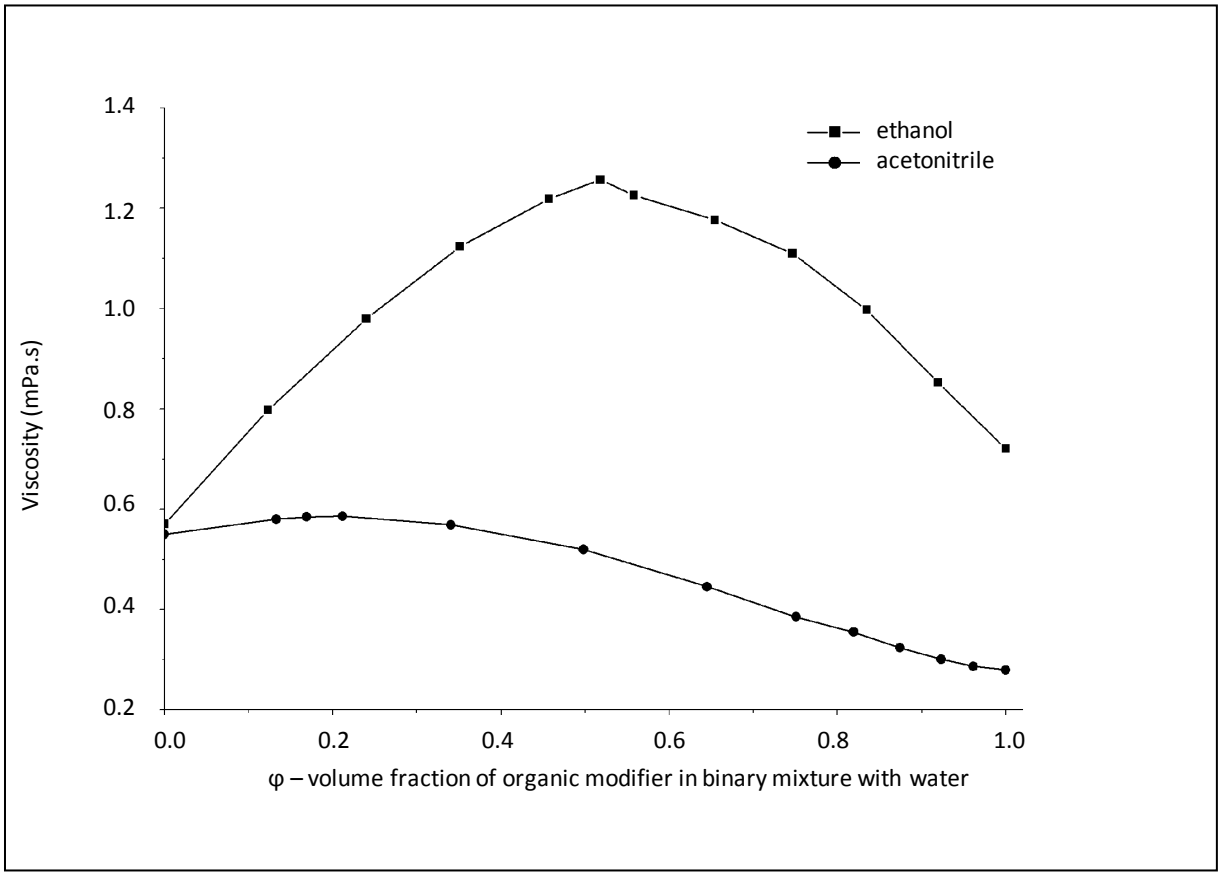

Fig. 5.2 Graphical representation of viscosities of ethanol and acetonitrile vs. volume fraction in the binary mixture with water at 50 °C. Data from [39] and [40].

In preliminary work the limitations of the maximum allowable column pressure drop, the suitable mobile phase flow rates and column temperatures were investigated. A series of measurements was done at different flow rate and temperature by using a 10 cm long segment combination of C18. The results are shown in Figure 5.3. The maximum length of segment combination in this work was 25cm for each stationary phase and the maximum allowable column pressure was 400 bar. In other words, the maximum column pressure is about 160 bar for a 10 cm long segment combination. The data in Figure 5.3 shows that the optimal chromatographic condition consists of the combination of a column temperature of 50 °C with a flow rate of 0.5 mL min^{-1}. Both the combinations of 30 °C with 0.5 mL min^{-1} and 50 °C with 0.55 mL min^{-1} resulted in a column pressure exceeding 160 bar at higher ethanol fractions. The combination of 50 °C with 0.45 mL min^{-1} was not employed as it was leading to longer analysis times.

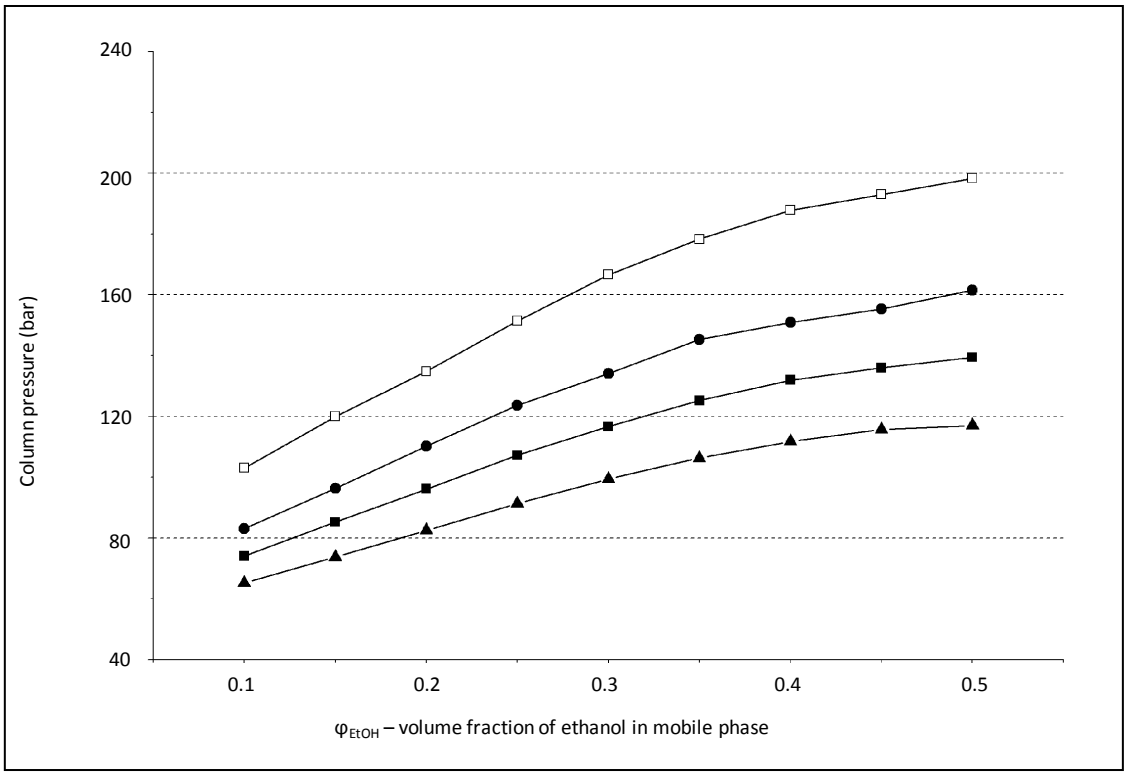

Fig. 5.3 Column pressure drop of a 10 cm C18 segment combination at different flow rates and column temperatures. Blank square (□): flow rate of 0.5 mL min^{-1} and column temperature at 30 °C; solid circle (●): flow rate of 0.55 mL min^{-1} and column temperature at 50 °C; solid square (■): flow rate of 0.5 mL min^{-1} and column temperature at 50 °C; solid triangle (▲): flow rate of 0.45 mL min^{-1} and column temperature at 50 °C.

An isocratic optimization with 15% (v/v) ethanol was first of all performed with the POPLC Optimizer of Bischoff Chromatography. The extra-column void time $t_{0,EC}$ was determined to be 0.045 min for uracil. The optimized combination of segments was thereby 1 cm C30 coupled with 4 cm C18 and 12cm C18EPS. The corresponding experimental chromatogram is shown in Figure 5.4. The total analysis time was about 110 min and the last eluting compound estriol (peak 9) shows a low detector response because of the large retention time. In addition, the selectivity of the critical pair, sulfadiazine (peak 4) and theophylline (peak 2), was unsatisfactory. This result shows that isocratic SOSLC is considerably limited in both analysis time and selectivity, especially when analyzing mixtures of compounds covering a large range of hydrophobicities [19]. Before the gradient SOSLC algorithm could be applied, the basic measurements for the retention models needed to be performed. The length of the segment combination for each stationary phase was thereby 10 cm. For each compound on each stationary phase, a series of isocratic analyses was performed on at least 5 levels of volume fraction of ethanol (see also Table 5.2). Note that compared to acetonitrile and methanol, ethanol has a higher eluotropic strength and therefore the scouting runs will be shorter. Additionally, for the same reason, less ethanol is likely to be required which is another “green” feature. In total 70 quadratic models (14 compounds on 5 stationary phases) were built based on the retention times

obtained in the basic measurements. The retention models constructed for the C18 phase are shown in Figure 5.1. The average regression correlation coefficient R^2 of the 70 models was 0.9998. This suggests that the experimental data fits well on the models and that reliable data prediction can be obtained for the gradient SOSLC application. The dwell time t_{dwell} in Eq. 5.9 was then determined to be 1.50 min at the flow rate of 0.5 mL min^{-1} and the dwell volume was therefore estimated to be 0.75 mL. A linear gradient, in which the volume fraction of ethanol increases from 10% to 50% (v/v) in 30 min, was used as a fixed gradient for the stationary phase optimization by applying the in-house developed software and the retention models. The combination of a column of 6 cm C18 and 12 cm C18EPS was proposed by the software as the optimal result. The predicted and the experimental separation are shown in Figure 5.5a and 5.5c, respectively. The corresponding retention times are given in Table 5.3.

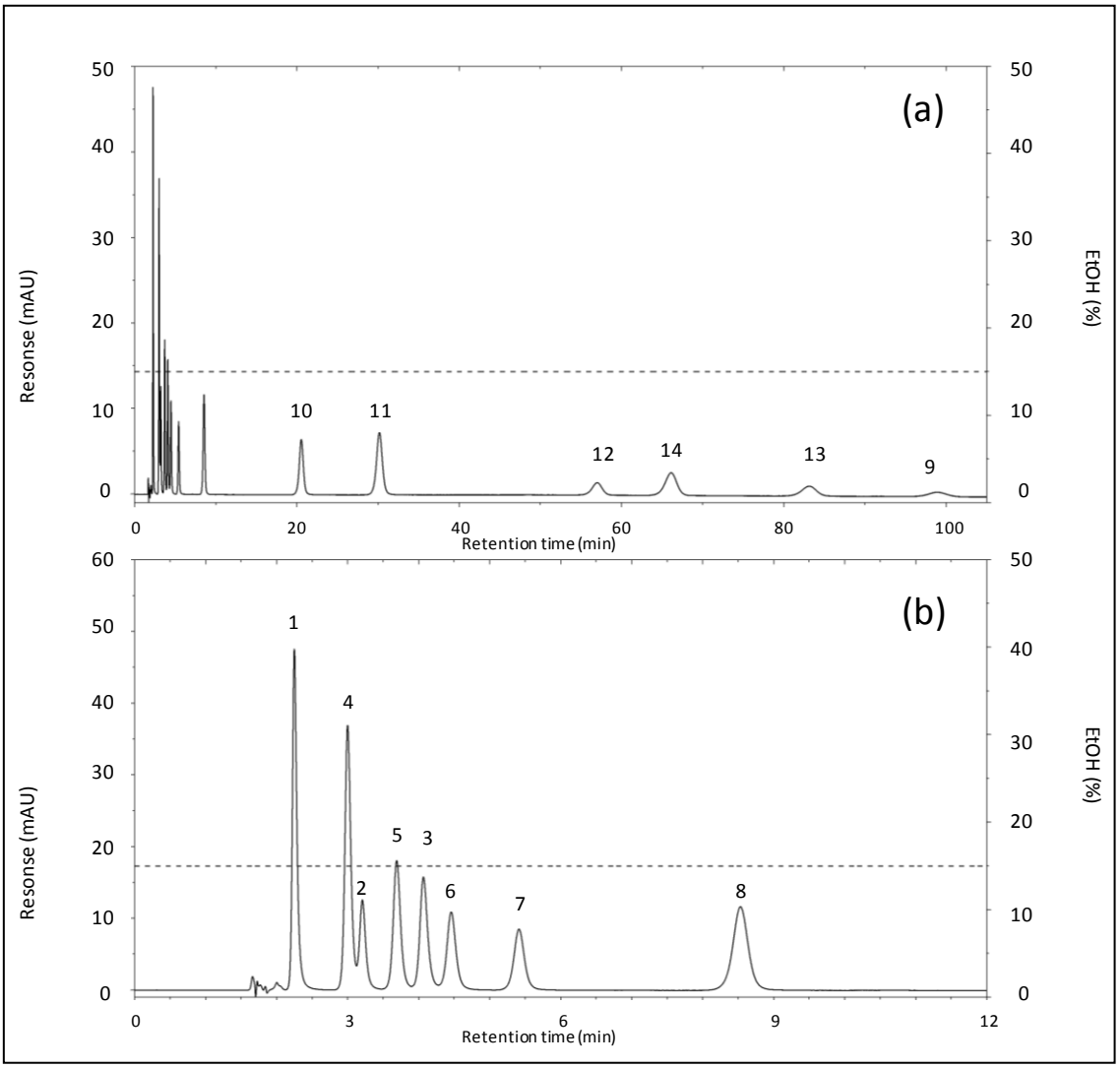

Fig. 5.4 Chromatogram of the isocratic optimization. Optimized segment combination: 1 cm C30, 4 cm C18 and 12cm C18EPS. (a) Full chromatogram; (b) zoomed section from 0 to 12 min. For peak identification, please refer to Table 5.1.

Table 5.3 Comparison of the predicted retention times with the uncalibrated algorithm for gradient analysis and the experimental retention time on the optimal segment combination: 6 cm C18 and 12cm C18EPS. Gradient: 10–50% v/v ethanol in 30 min. For peak identification, see Table 5.1.

Numbering	Predicted retention time (min)	Observed retention time (min)	Relative deviation (%)
1	3.224	3.212	0.37
2	4.897	5.075	−3.51
3	6.292	6.628	−5.07
4	4.381	4.458	−1.73
5	5.588	5.804	−3.72
6	6.816	7.126	−4.35
7	7.728	8.210	−5.87
8	10.269	10.902	−5.81
9	22.382	23.308	−3.97
10	14.805	15.694	−5.66
11	16.728	17.662	−5.29
12	19.192	20.005	−4.06
13	21.045	21.904	−3.92
14	19.802	20.626	−3.99

It is clear from these results that the predicted top ranked column combination is indeed resulting into baseline separation of all analytes and that the expected elution order and selectivities of the various peaks are correctly reflected in the confirmatory experiments. It can, however, also be seen that the predicted retention times for most compounds underestimate the experimental results. The maximal discrepancy in retention time comprises about 6% in this example (peak No. 7 in Fig. 5). Other, multi-linear, gradient profiles were also investigated which are given in Table 5.4. The corresponding predictions and experimental confirmations are shown in Table 5.5. The errors in prediction thereby run up to 19% in gradient 2 in Table 5.4, whereby a steep gradient is used in the beginning of the chromatogram. In all cases, however, the predicted column combination presented a solution leading to baseline separation of all peaks in the experimental chromatograms. The reason for this is that the software is ranking the signals according to the maximal retention time difference for every signal. It appears that the resulting over-resolution of the peaks in the chromatogram is such that it is difficult to undo by the observed discrepancies between prediction and experiment. Additionally as in all cases the shifts are quite systematic (all peaks shift either to the left or to the right in a particular region of the chromatogram). Effective overlap of 2 peaks during the experiments contrary to a prediction is not taking place with the analyzed samples.

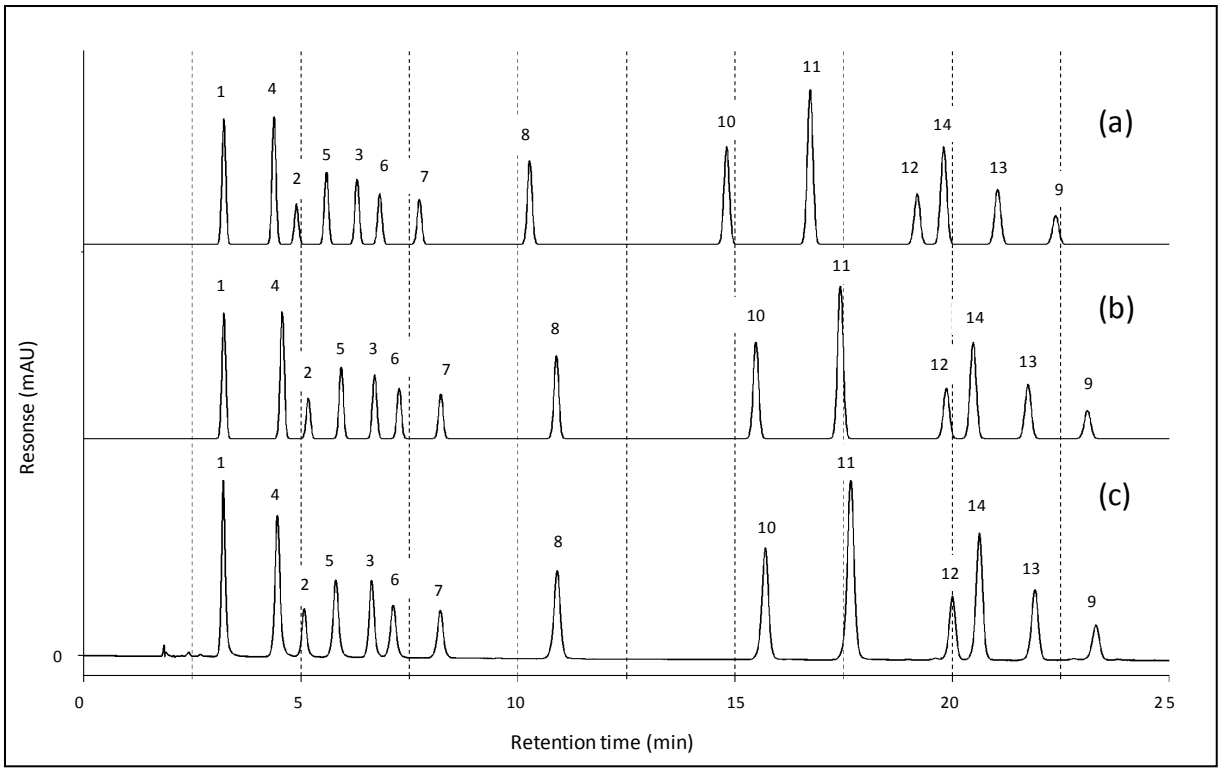

Fig. 5.5 Comparison of predicted and experimental chromatograms on the optimal segment combination: 6 cm C18 and 12cm C18EPS. (a) Predicted chromatogram with the algorithm as such; (b) predicted chromatogram with the empirical calibration procedure; (c) experimental chromatogram.

The reliability of the top ranked predictions that they do deliver good separation of all the analytes is illustrated in Table 5.6 and Figure 5.6.

Table 5.4 Investigated multi-linear gradient profiles.

No.		Gradient profile			
1	Time (min)	0	7	29	64
	φEtOH (v/v)	10%	10%	29%	32%
2	Time (min)	0	1	8	26
	φEtOH (v/v)	0%	11%	28%	30%
3	Time (min)	0	6	7	86
	φEtOH (v/v)	4%	17%	29%	34%

Table 5.5 Comparison of the predicted retention times obtained from the uncalibrated and calibrated algorithms with the observed retention times. For the gradient profile details, see Table 5.4. Compound identification, see Table 5.1.

No. gradient profile	Numbering of compounds	Observed Rt (min)	Without calibration		With calibration	
			Predicted Rt (min)	Relative deviation (%)	Predicted Rt (min)	Relative deviation (%)
1	1	3.890	4.000	2.83	4.000	2.83
	4	4.748	5.052	6.40	5.052	6.40
	2	5.721	6.036	5.51	6.036	5.51
	5	6.328	6.861	8.42	6.861	8.42
	6	8.410	9.292	10.49	9.292	10.49
	3	9.411	10.195	8.33	10.195	8.33
	7	10.194	11.040	8.30	11.078	8.67
	8	12.848	12.393	-3.54	13.293	3.46
	10	15.118	14.339	-5.15	15.127	0.06
	11	16.514	15.727	-4.77	16.424	-0.54
	12	18.746	18.461	-1.52	18.899	0.82
	14	19.466	19.288	-0.91	19.696	1.18
	13	20.812	20.641	-0.82	21.037	1.08
	9	22.934	22.473	-2.01	22.936	0.01
	Average	-	-	**4.93**	-	**4.13**
2	1	5.780	4.683	-18.98	5.622	-2.73
	4	6.445	5.336	-17.21	6.250	-3.03
	2	6.808	5.692	-16.39	6.533	-4.04
	5	7.231	6.146	-15.00	6.994	-3.28
	3	7.613	6.522	-14.33	7.297	-4.15
	6	7.942	6.895	-13.18	7.716	-2.85
	7	8.596	7.531	-12.39	8.347	-2.90
	8	10.163	9.111	-10.35	9.985	-1.75
	10	12.780	11.705	-8.41	12.385	-3.09
	11	14.436	13.294	-7.91	13.904	-3.69
	12	16.474	15.412	-6.45	15.937	-3.26
	14	17.349	16.279	-6.17	16.792	-3.21
	13	19.503	18.379	-5.76	18.877	-3.21
	9	23.370	21.954	-6.06	22.453	-3.92
	Average	-	-	**11.33**	-	**3.22**
3	1	5.965	5.156	-13.56	5.732	-3.91
	4	7.044	6.240	-11.41	6.814	-3.27
	2	7.722	6.828	-11.58	7.584	-1.79
	5	8.406	7.586	-9.75	8.315	-1.08
	3	9.026	8.172	-9.46	9.069	0.48
	6	9.550	8.768	-8.19	9.581	0.32
	7	10.427	9.601	-7.92	10.579	1.46
	8	11.457	10.550	-7.92	11.834	3.29
	10	13.493	12.546	-7.02	13.114	-2.81
	11	15.010	14.058	-6.34	14.349	-4.40
	12	16.463	15.755	-4.30	16.216	-1.50
	14	17.190	16.543	-3.76	17.013	-1.03
	13	19.096	18.532	-2.95	18.976	-0.63
	9	22.937	22.472	-2.03	21.812	-4.90
	Average	-	-	**7.59**	-	**2.20**

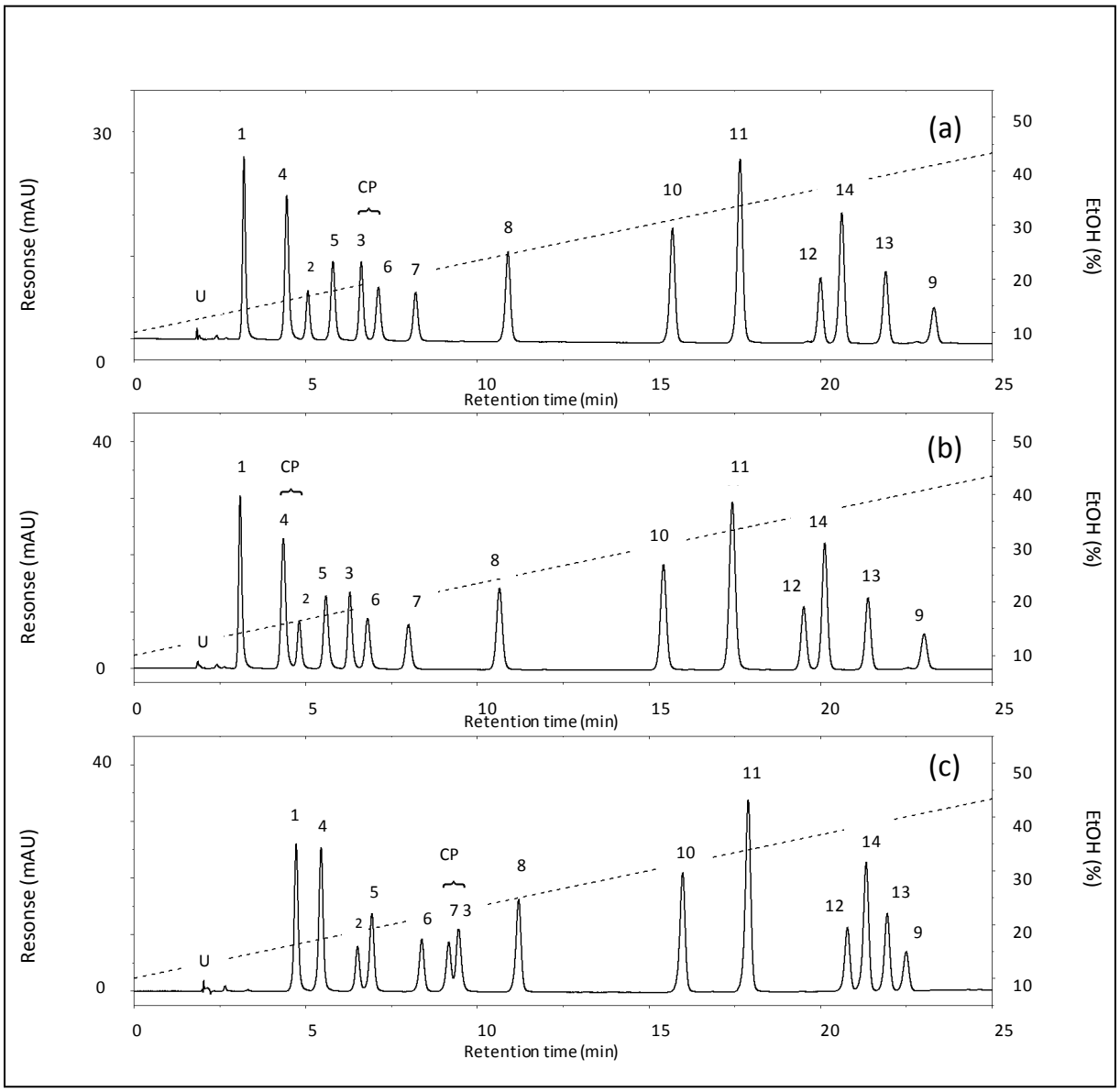

Fig. 5.6 Experimental chromatograms on the segment combinations obtained in gradient optimization. (a) Chromatogram of segment combination of rank 1; (b) chromatogram of segment combination of rank 20; (c) chromatogram of segment combination of rank 50. CP means critical pair. For detail description, please refer to Table 5.6. For peak identification, please refer to Table 5.1.

The chromatograms obtained with the first, twentieth and fiftieth ranked suitable column combination is thereby shown and it can be seen that only in the latter case the resolution between a critical pair starts to be affected. In Figure 5.6a and 5.6b all 14 compounds are baseline separated in 25 min. Compared with the results obtained by isocratic optimization (Fig. 5.4), the analysis time was considerably shortened and the selectivity of the critical pair was improved in the gradient

optimization. This demonstrates that the use of SOSLC with green mobile phases can achieve comparable separations to what can be obtained by using acetonitrile.

In order to improve the accuracy of the retention time predictions a survey of plausible causes was performed. HPLC hardware issues due to inaccurate flow rates, mixing or compressibility settings could be excluded suggesting inaccuracies in the numerical approach. The most probable cause of the observed discrepancies is thereby the void time measurements. Even very small errors in void time measurement can thereby eventually lead to the observed discrepancies between prediction and experiment. In this study the time counter (Δt) was set at 1 s. Therefore a late eluting peak such as peak 9 in Figure 5.5a, went through 1343 iterative cycles whereby a small error can be continuously accumulated. The fact that for a constant linear gradient the errors mostly increase with increasing retention time sustains this hypothesis. This can also explain the deviations observed in the multi-linear gradients. In gradient 1 in Table 5.5, the discrepancies between prediction and experiment in the first 7 minutes do not exceed the shifts observed when using the POPLC kit in the conventional isocratic way [41]. The gradient applied from then on (including dwell time) leads again to negative residuals which are similar to what was observed in Figure 5.5 and Table 5.3. Comparable effects are visible in gradients 2 and 3 in Table 5.5. It appears that the steep gradient in the beginning of gradient 2 is also leading to high discrepancies. It can thereby be argued if a time counter (Δt) of 1 s is sufficiently small for a fast gradient as applied in that section of the chromatogram.

However, no single void marker is truly unretained over a broad range of organic modifier fractions, especially when various stationary phases are used. Uracil is somewhat retained under RP-LC conditions at low (and very high) modifier fractions and depicts a minimum around 50% modifier and is accepted as a suitable void marker for C18 and C18EPS stationary phases [42]. Although it could be expected that salts such as KI and NaNO2, which can be detected at low UV wavelengths, are a better choice compared to small, polar organic molecules, it appears that salts are also retained due to various ion exchange and exclusion phenomena with residual silanol functions on the stationary phase [43]. The retention of KI (100 mM, 5 µL injection) vs. uracil is shown in Figure 5.7 for the ethanol fractions used in the present study on a C18EPS segment. As it is clear that uracil is preferable for most conditions compared to the salt, the former was used as t_0 marker in this study. In the proposed algorithm, quadratic models where therefore built to estimate and predict the retention of uracil for each stationary phase. As the average regression coefficient R^2 for uracil's quadratic models on the five stationary phases was 0.990, which is worse than that of the models for the compounds, it might explain the observed deviation between experiment and prediction.

Table 5.6 The predicted segment combinations in gradient optimization and the corresponding critical pair, predicted and observed retention time differences. Δt_r is the retention time difference between the peaks of the critical pair. For peak identification, please refer to Table 5.1.

Rank	Segment combination	Critical pair	Predicted Δt_r (min)	Observed Δt_r (min)
1	6cm C18 + 12 cm C18EPS	No. 3, No. 6	0.564	0.498
20	5 cm C18 + 12 cm C18EPS+1cmCN	No. 4, No. 2	0.459	0.464
50	8 cm C18EPS+5cmC30+5cmphenyl	No. 7, No. 3	0.252	0.287

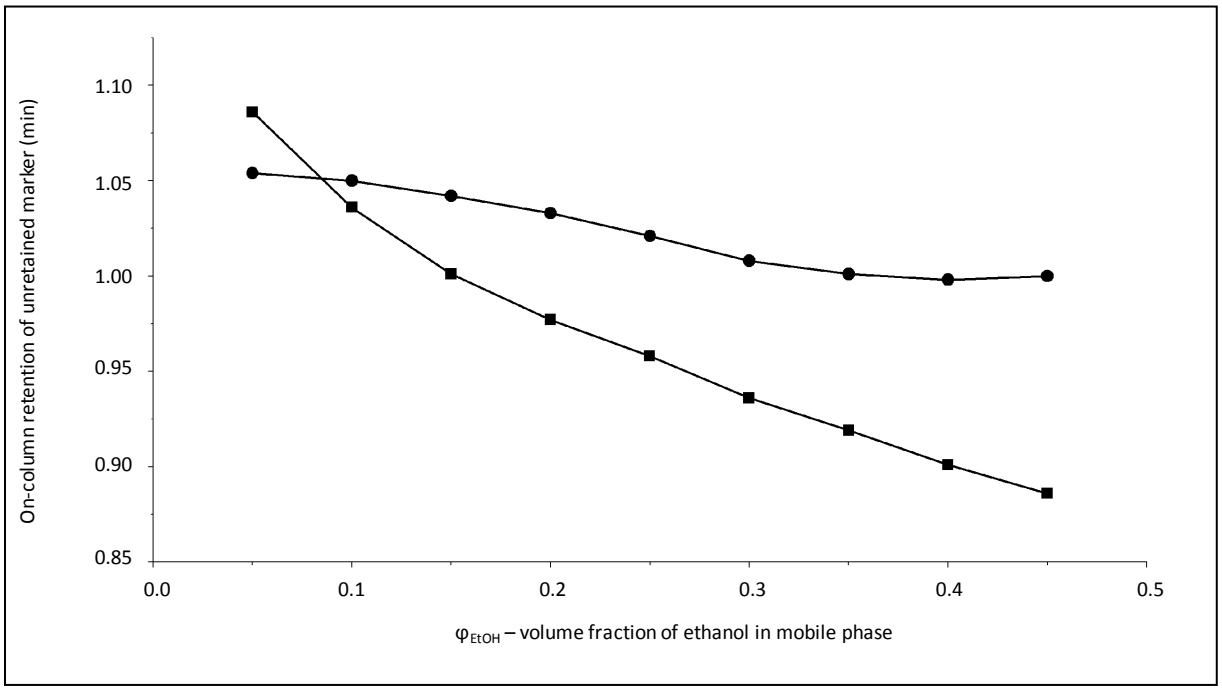

Fig. 5.7 On-column retention times of uracil and KI used as unretained markers on 10 cm C18EPS segment column at a flow rate of 0.5 mL min^{-1} and a column temperature at 50 °C. Solid square (■): uracil; solid circle (●): KI.

In order to improve the accuracy of retention time prediction, an empirical calibration is proposed here which acts on the migrated distance of analyte band in the numerical integration. A calibration coefficient is therefore introduced in Eq. 5.11 leading to

$$d_{m,updated} = d_m + \frac{1}{1+ps} u \frac{1}{1+k} \Delta t \qquad \text{(Eq. 5.13)}$$

where s is the concurrent slope of the gradient curve (the instant increment of the volume fraction φ per second) and p is the empirical constant coefficient with the unit of time in seconds.

The empirical constant p is obtained by minimizing the prediction deviation of two selected compounds' retention times under the given gradient (10–50% (v/v) in 30 min). In this chapter, for example, the experimental retention times of sulfadiazine (No. 4) and estriol (No. 9) under the linear gradient profile were compared with those predicted and the empirical constant p was gradually tuned to be 8 s.

To verify the calibration, the predicted chromatogram of the sample mixture from the calibrated algorithm is shown in Figure 5.5b and overlaid with the predicted chromatogram from the uncalibrated algorithm (Fig. 5.5a) and the experimental chromatogram (Fig. 5.5c). A significant improvement of prediction accuracy can be observed. For multiple-linear gradients shown in Table 5.4, the prediction deviation after calibration is shown in Table 5.5 which also demonstrates the improvements in prediction which can be obtained by making use of this empirical calibration procedure.

Compared to established predictive software packages such as DryLab®, gradient SOSLC method currently requires a considerable number of basic measurements. The use of LC–MS to track all peaks simultaneously should, however, solve this problem as this should result in 5 or 6 runs per column (for each organic modifier fraction) for each stationary phase. The columns can also be imagined in a system equipped with automatic column selection which should allow unattended SOSLC optimization. Note that SOSLC makes maximal use of the orthogonality of the selectivity from the different stationary phases. This approach has proven to offer many practical solutions for the analysis of complex samples before [44]. Although some commercial software allow the isocratic SOSLC option (making use of Eq. 5.8), the gradient approach described here is not included in other predictive software packages. Therefore, gradient SOSLC can be considered as a new tool in the family of in-silico method development approaches which is offering promising prospects in the field of green chromatography.

5.5 Conclusions

Gradient SOSLC has been extended to green chromatography. Taking into consideration the high viscosity of ethanol, a flow-rate of 0.5 mL min^{-1} and a column temperature of 50 °C were used as operation conditions. A numerical integration based on the quadratic retention models was applied to predict the analytes' retention times under gradient conditions. In addition, an empirical calibration is proposed to correct the predicted retention time shift probably caused by the inaccurate measurements of void time and accumulative error in the numerical iteration process under gradient conditions. As a result, the optimized segment combination for a given gradient profile can lead to a fast and full baseline separation, showing competitive quality of separation to what can be obtained when using acetonitrile. Green SOSLC can therefore help in fundamentally changing the way in which HPLC methods are developed: first preselecting a green mobile phase and then optimizing the stationary phase.

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Chapter 6: Improved pharmaceutical impurity determinations by HPLC with innovative high efficiency SOSLC strategies

Summary

Stationary phase optimized selectivity liquid chromatography (SOSLC) is a reversed-phase LC (RP-LC) approach to optimize the selectivity for a given separation by combining a set of different stationary phases in a multi-segment column. Depending on the sample to be analyzed, an SOSLC optimized separation method can be obtained under isocratic, step-wise gradient and linear gradient mode as described in Chapter 3, Chapter 4 and Chapter 5, respectively. The fundamentals and features of SOSLC have been described and demonstrated in these previous chapters. In the field of pharmaceutical analysis, selectivity is a particulary important issue for separating impurities from the active pharmaceutical compound (API), which is typically characterized by a broad overloaded peak. Overlap with an impurity is a problem that is often observed during method development. In this chapter, the benefits of the SOSLC approach for optimizing the selectivity in combination with the use of extended column lengths for increasing the efficiency are described. This combination of both selectivity and increased efficiency leads to a significantly improved resolution in a way that is impossible with only the individual optimization. This is demonstrated by means of two simulated but representative pharmaceutical test mixtures whereby API and impurity simulation is performed.

6.1 Introduction

As discussed in Chapter 2, an alternative option for attempting a sufficient separation in the field of HPLC is to increase the resolution by increasing the efficiency through the use of an extended column length (see eq. 2.4 and eq. 2.6). The relation with elevated temperatures, leading to a decrease of viscosity and backpressure, and the link towards the van Deemter curve [1,2] have been discussed in detail in sections 2.5.4, 2.6, 2.7.2.5 and 2.7.2.6.

According to the resolution equation (Eq. 2.10) an increase of N by for exampling doubling the column length results in an increase of the resolution with a factor √2. This approach can be eligible for resolving a peak pair which is straggling with an almost baseline separation. However, this approach can remain elusive in the case whereby two peaks are quasi completely overlapping due to an intrinsic lack of selectivity provided by the operational chromatographic parameters. As an objective in this chapter, an attempt is made to combine the strategy of increased efficiency through extended column lengths with a selectivity optimization tool.

As discussed the chapters before, employment of stationary phase optimized selectivity liquid chromatography (SOSLC) whereby the stationary phase becomes a tunable parameter by connecting column segments, with variable lengths, of different stationary phases can be considered as an innovative selectivity optimization tool [3-9]. Because the separation in SOSLC is essentially delivered by the stationary phase optimization, selectivity optimization based on the optimization of the mobile phase and of other tunable parameters is thereby becoming less important. As a consequence, more liberty remains concerning the mobile phase compounds. In this way, SOSLC offers the possibility to perform green chromatography as described in Chapter 5[10].

Originally, an SOSLC optimization procedure and algorithm was introduced to be applicable for isocratic conditions. This approach is based on the PRISMA model for optimization of the mobile phase in LC [27-29]. Due to the limitation to isocratic analysis, whereby this approach is inadequate for mixtures of compounds that differ significantly in polarity and hydrophobicity, an SOSLC multiple step gradient strategy was developed and described in Chapter 3 [11]. Although useful, this step gradient method is characterized by some limitations such as the need for arbitrary choices, an undefined order in which the different segments must be coupled and only roughly estimated retention times. For a more practical and flexible approach, a renewed optimization approach and general prediction algorithm, based on numerical integration, were developed and described in Chapters 4 and 5, allowing the use of linear gradient mode as well [12]. Furthermore, arbitrary decisions about the classification of sample compounds are eliminated, and accurate predictions of

all retention times and selectivity factors are provided as well. It has to be understood that when a baseline separation for multiple analytes is wanted, an SOSLC optimized column segment combination offers a compromise solution for all peak pairs whereby the selection of this optimal combination is finally based on the highest achievable selectivity for the most critical peak pair. On one side, due to the presence of multiple critical peak pairs in a sample, it can occur that no full baseline separation is obtained for one or a few peak pairs with the SOSLC optimization approach only. On the other side, using the extended column length approach but with only a single stationary phase, does not always offer the wanted baseline separation as well.

In this chapter, the benefits of combining SOSLC with the use of extended column lengths are investigated and demonstrated with two representing test mixtures. Active pharmaceutical ingredient (API) and impurity are hereby simulated as an application to demonstrate the improved resolution in the case of the presence of a broad overloaded API peak. The separation of each mixture is firstly optimized by means of the linear gradient SOSLC optimization method which is described in Chapter 4. Subsequently, the strategy of the work in this chapter consists of multiplying the length of the obtained optimal SOSLC columns leading to a higher efficiency but displaying identical selectivity. The corresponding gradient profile is thereby also adapted to the length of the column.

6.2 Experimental

6.2.1 Materials

Mixture 1: Butyl-4-hydroxybenzoate, ethyl-4-hydroxybenzoate, benzophenone, acetophenone, propiophenone, n-butyrophenone, 4-ethylbenzoic acid, 4-butylbenzoic acid, sulfamethoxazole, sulfamerazin, sulfamethizol, prednisolone, metamitron, metribuzin, atrazin-desethyl and aldicarb.

Mixture 2: prednisolone, methylprednisolone, methylboldenone, methyltestosterone, triamcinolone acetonide, cortexolone, cortisone and testosterone.

All analytes were purchased from Sigma-Aldrich (Bornem, Belgium). The HPLC solvents water, methanol (MeOH), acetonitrile (ACN) and formic acid (FA) were purchased from Biosolve (Valkenswaard, The Netherlands). Stock solutions of all compounds were prepared in acetonitrile (ACN) with a concentration of 40 mg mL^{-1}. Model mixtures of these compounds were prepared by diluting the stock solutions with 50/50 water/methanol (MeOH) to a final mixture whereby the compounds have a concentration of 30 µg mL^{-1}. For impurity analysis simulations, mixture 1 was prepared with a concentration of 40 mg mL^{-1} for atrazin-desethyl and a concentration of 20 µg mL^{-1}

for all other compounds, in order to simulate a 0.05% impurity analysis. Mixture 2 was prepared with a concentration of 2 mg mL^{-1} for cortisone and 1 µg mL^{-1} for the other compounds.

Two POPLC Basic Kits 250-5 from Bischoff Chromatography (Leonberg, Germany) were combined in this study. From each kit, following stationary phases were used: ProntoSIL C18 SH 2 (a C18 stationary phase with all classic features), ProntoSIL C18 EPS 2 (a C18 stationary phase with embedded polar amide groups) and ProntoSIL C30. For each of these phases, column segments of 10 (x2), 20 (x2), 40 (x4), 60 (x2) and 80 (x2) mm were available. The stationary phases all have a particle size of 5 µm and the column segments have an internal diameter of 3 mm. Robust columns composed of combinations of these segments can be assembled by means of POPLC connecting pieces.

An Agilent 1100 HPLC system equipped with DAD-detector was used for mixture 1 and an Agilent 1200 HPLC system also equipped with DAD-detector was used for mixture 2 (Agilent Technologies, Waldbronn, Germany).

6.2.2 Methods

Concerning mixture 1, the mobile phase was composed of water (0.1 % formic acid (FA)) and MeOH. Detection was performed at 230 nm. The injected volumes were 2 µL, except for the impurity analysis simulation for which the injection volume was 5 µL. The flow rate was set at 0.5 mL min^{-1}. A fixed operating temperature of 60 °C was set up and maintained by means of a Polaratherm oven, series 9000 (SandraSelerity Technologies Inc., Salt Lake City, USA). Concerning mixture 2, the mobile phase was composed of water and ACN. Detection was performed at 230 nm. The injection volumes were 2 µL, except for the impurity analysis simulation for which the injected volume was 8 µL. The flow rate was set at 0.5 mL min^{-1}. All experiments concerning to mixture 2 were performed at room temperature.

Chemstation software (Agilent Technologies) was used for retention data collection. Linear Gradient POPLC optimizer – an in-house made software package based on the fundamentals described in Chapter 4 – was used for optimization calculations and predictions.

6.3 Results and discussion

As mentioned in section 6.1, two simulated but representing test mixtures are used to evaluate the strategy of combining SOSLC with extended column lengths leading to a combined boost of selectivity and efficiency. First the separation of the two mixtures, containing analytes at equal non-overloaded concentration levels, are optimized with linear gradient SOSLC as described in Section 4. Subsequently, depending on the obtained insufficient resolution due to the presence of multiple critical peak pairs but of which the intrinsic selectivity is tuned as optimal as possible by SOSLC, the column length extension is implemented for boosting the resulting resolution. At last, a simulation whereby the concentration of a compound is altered to a typical API level and whereby the remaining analytes are scaled to impurity levels, is performed fur further demonstration of the benefits of this concept.

As mentioned before, the first step in linear gradient SOSLC method development is the retention profiling of the compounds as a function of the organic modifier concentration. Therefore, initial basic measurements have to be performed at multiple isocratic stages. A minimum of 3 such basic measurements for each analyte on each stationary phase is necessary as a quadratic relationship between the logarithm of the retention factor *ln(k)* and the organic modifier concentration φ is used for retention modeling (Eq. 2.15).

Note that the more equidistant the measuring isocratic levels are, the more reliable is the obtained retention profile curve. Note as well that it makes no sense for the very apolar analytes to perform profiling measurements at high aqueous isocratic levels where excessive retention times are faced. A data point measurement at a high aqueous level can be missed and replaced by extrapolation of the experimental retention profile curve because the migration velocity and distance of a very apolar compound at a high aqueous level, during a gradient run, is small and almost negligible.

6.3.1 Increased efficiency SOSLC of mixture 1

The basic measurements of the compounds of mixture 1 (see Table 6.1 for compound numbers for peak identification) on the different stationary phases are performed at 8 different equidistant isocratic stages between 80/20 and 10/90 water (0.1 % FA)/MeOH. A constant temperature of 60 °C was hereby applied. The obtained experimental retention times of the basic measurements were then incorporated in the Linear Gradient POPLC Optimizer software. For further optimization, a generic linear gradient profile of water (0.1 % FA)/MeOH going from 80/20 at minute 0 to 10/90 in 30 minutes was set up in the software. During the calculation processing, the different column segment

combinations up to 25 cm are screened. All the retention times are hereby calculated as well as a chromatographic response function which is hereby the difference between consecutive retention times of the eluting analytes. Finally, the highest ranked column segment combination was obtained according to its highest value of the applied chromatographic response function for the most critical peak pair on each combination. A maximum analysis time limit filter was set up at 30 min.

An optimal combination consisting of 2 cm C30 at the column inlet, followed by 17 cm C18 EPS 2 and ending with 6 cm C18 SH 2 (25 cm in total) was found. A simulation of the chromatogram is shown in Figure 6.1. It can be noticed that the optimal SOSLC column for this separation can be considered as a compromise as there are multiple critical peak pairs present in the mixture that elute very close according to the prediction. The experimental chromatogram on this optimal SOSLC column is shown in Figure 6.2A whereby the selectivity matches the prediction. The presence of the multiple critical peak pairs is confirmed as they are not completely resolved to the baseline in the experimental chromatogram.

Table 6.1 List of compounds – numbered for identification – of mixture 1.

Compound number	Compounds of mixture 1
1	Butyl-4-OH-benzoate
2	Ethyl-4-OH-benzoate
3	Benzophenone
4	Acetophenone
5	Propiophenone
6	n-butyrophenone
7	4-ethylbenzoic acid
8	4-butylbenzoic acid
9	Sulfamethoxazole
10	Sulfamerazin
11	Sulfamethizol
12	Prednisolone
13	Metamitron
14	Metribuzin
15	Atrazin-desethyl
16	Aldicarb

As the purpose of this set-up is to improve the resolution by extension of the optimized SOSLC column length, the length was doubled resulting in a total column of 4 cm C30, followed by 34 cm C18 EPS2 and ending with 12 cm C18 SH2 (50 cm in total). The time period of the corresponding linear gradient profile is doubled as well, starting at water (0.1 % FA)/MeOH 80/20 at minute 0 and

going to 10/90 in 60 min. The experimental chromatogram on this prolonged optimal column is shown in Figure 6.2B. A clear improvement of resolution is visible whereby all peaks are now separated to the baseline. Note that the profile of the chromatogram and overall selectivity remains maintained compared to the original 25 cm optimized SOSLC column. A comparing analysis on a 50 cm 'pure' C18 SH2 column under the same conditions is shown in Figure 6.2C, whereby Et-4-hydroxybenzoate and Metribuzin are still overlapping which illustrates the statement that only increasing efficiency can remain elusive.

6.3.2 Increased efficiency SOSLC of mixture 2

For mixture 2 (see Table 6.2 for compound numbers and peak identification), the same SOSLC optimization procedure was performed. Room temperature was applied as working temperature. The basic measurements of all the compounds on the different stationary phases are performed at the following 3 isocratic stages: 80/20, 50/50 and 90/10 water/ACN. The obtained experimental retention times of the basic measurements were then incorporated in the Linear Gradient POPLC Optimizer software. For further optimization, a generic linear gradient profile of water/ACN going from 80/20 at min 0 to 10/90 in 25 min was set up. A maximum analysis time limit was set up at 15 min. The obtained optimal combination consists of 6 cm C30 connected with 9 cm C18 SH 2 (15 cm in total). A simulation of the chromatogram is shown in Figure 6.3A. The experimental chromatogram on this optimal SOSLC column is shown in Figure 6.3B whereby it can observed that the profiles and selectivity of both simulation and experimental chromatograms match well and moreover baseline separation is obtained. A multiplication of the column length with a factor 3 was still achievable for remaining under a backpressure of 400 bar, resulting in a column of 45 cm (18 cm C30 connected with 27 cm C18 SH 2) that offers an increased efficiency and resolution as shown in Figure 6.3C and compared to Figure 6.3B. In correspondence, the gradient profile was adapted and starts from 80/20 water/ACN at minute 0 and goes to 10/90 in 75 min. The 45 cm optimized column is shown in Figure 6.4.

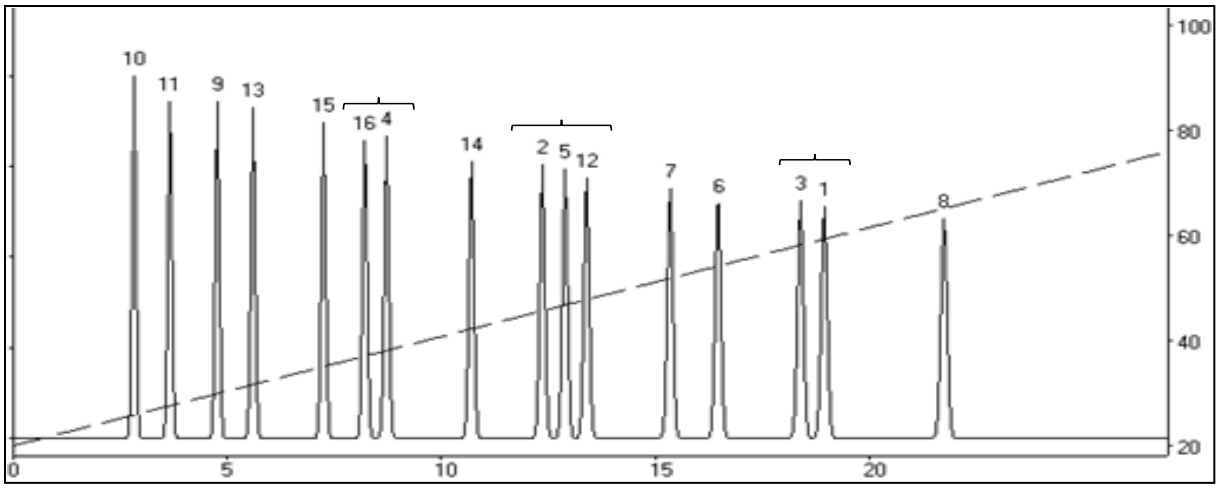

Fig. 6.1 Simulation chromatogram for mixture 1 by the Linear Gradient POPLC Optimizer software. Found optimal column: 2 cm C30 + 17 cm C18 EPS2 + 6 cm C18 SH2. Gradient profile: water (0.1%FA)/MeOH: 80/20 → 10/90 in 30 min. Peak identification: see Table 6.1.

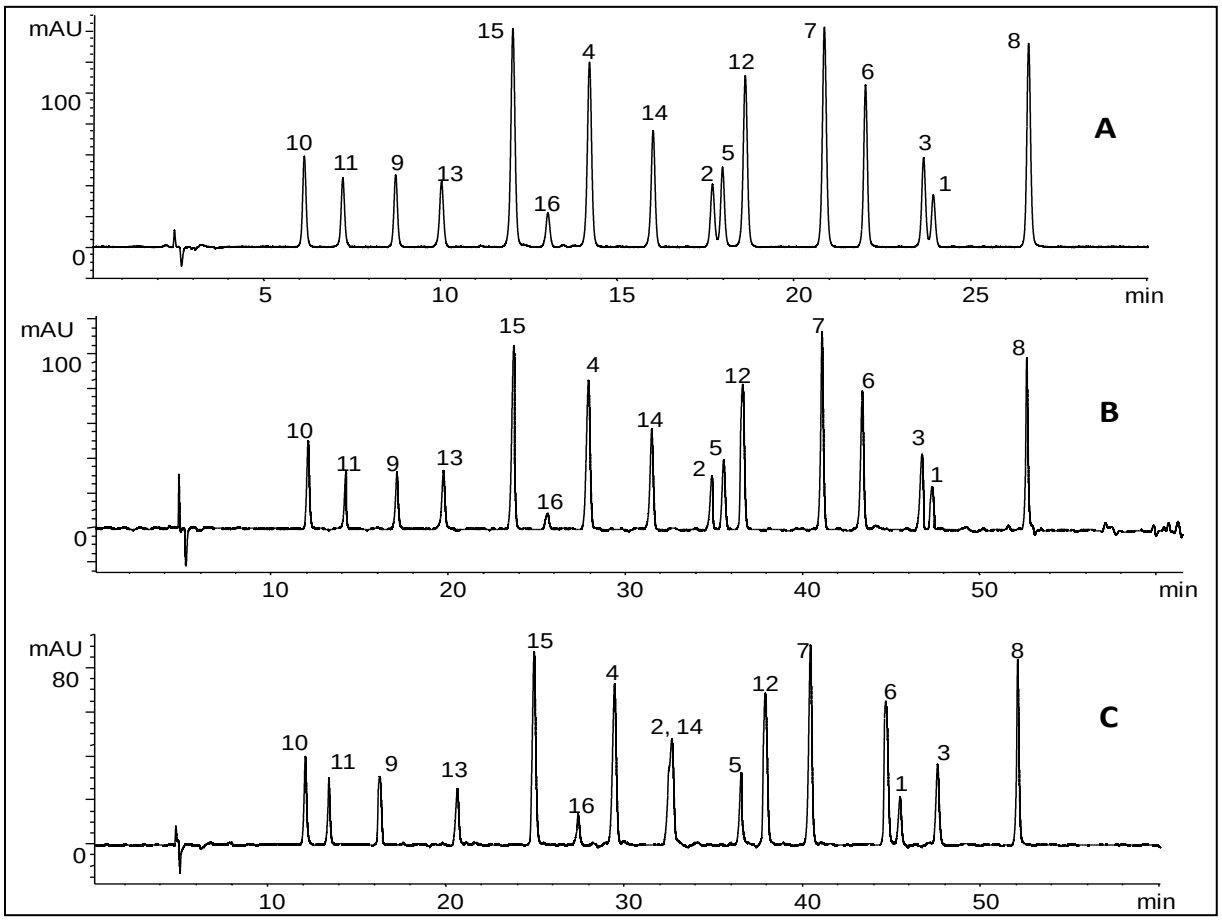

Fig 6.2 Mixture 1: A: Experimental chromatogram on the optimal SOSLC column (2 cm C30 + 17 cm C18 EPS2 + 6 cm C18 SH2; total 25 cm). B: Experimental chromatogram on the extended optimal SOSLC column with double length (4 cm C30 + 34 cm C18 EPS2 + 12 cm C18 SH2; total 50 cm). Gradient profile: water(0.1% FA)/MeOH: 80/20 → 10/90 in 60 min. C: Experimental chromatogram on a 50 cm C18 SH2 column. Gradient profile: water(0.1% FA)/MeOH: 80/20 → 10/90 in 60 min. See Table 6.1 for peak identification.

Table 6.2 List of compounds – numbered for identification – of mixture 2.

Compound number	Compounds of mixture 2
1	Me-prednisolone
2	Me-boldenone
3	Me-testosterone
4	Triamcinolone acetonide
5	Cortexolone
6	Cortisone
7	Prednisolone
8	Testosterone

When the concentration of one compound is intensively scaled up or when an API is present, a significant band broadening is observed when remaining compounds or impurities at levels above 0.05% area of the peak normalization have to be detected as well. Developing a separation method on the conventional approach is often not an obvious task as the broad API band has to be separated from the compounds eluting before and behind. How increased efficiency SOSLC thereby can serve as a solution tool, is described in section 6.3.3.

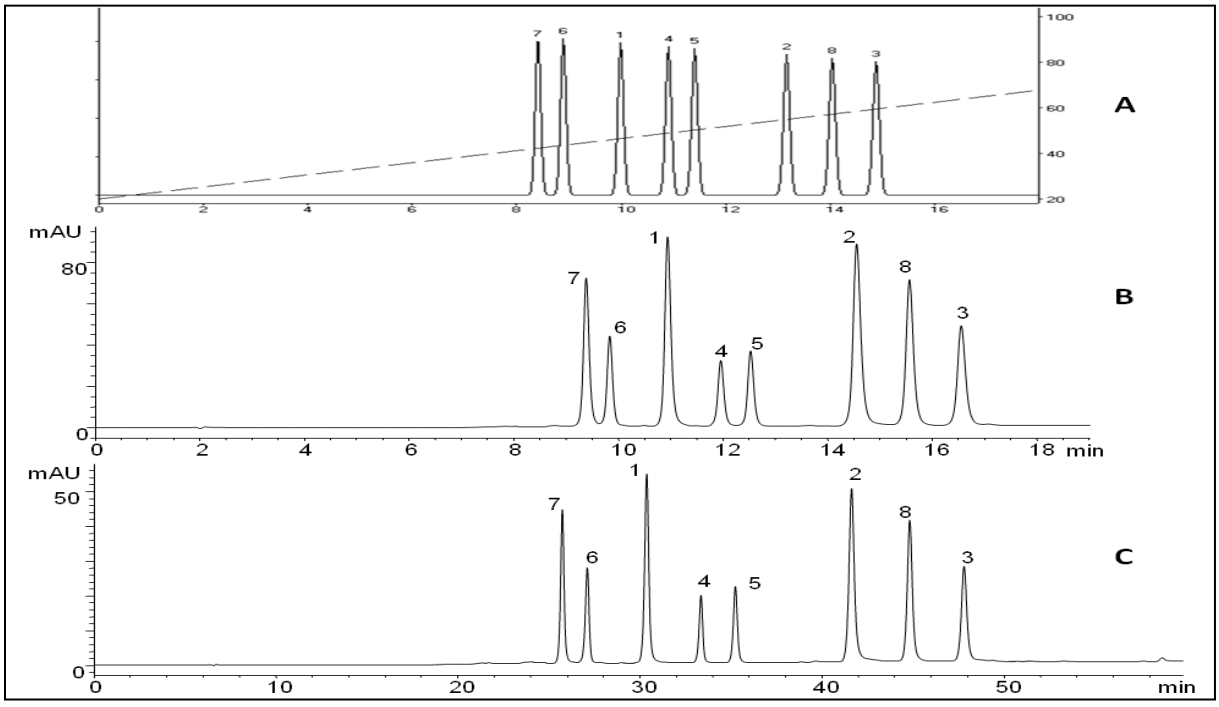

Fig. 6.3 Mixture 2: A: Simulation chromatogram for mixture 2 by the Linear Gradient POPLC Optimizer software. Found optimal column: 6 cm C30 + 9 cm C18 SH2. Gradient profile: water/ACN: 80/20 → 10/90 in 25 minutes. B: Experimental chromatogram on the optimal SOSLC column (6 cm C30 + 9 cm C18 SH2). C: Experimental chromatogram on the extended SOSLC optimized column with triple column length (18 cm C30 + 27 cm C18 SH2). Gradient profile: water/ACN: 80/20 → 10/90 in 75 minutes. See table 6.2 for peak identification.

Fig. 6.4 Picture of the 45 cm high efficiency SOSLC optimized column for mixture 2.

6.3.3 API and impurity level simulation

In the pharmaceutical industry, analysis of drug substances and drug products is from utmost importance. Worldwide, authorities set high standards for the pharmaceutical industry and demand a high quality for drugs in order to be approved and to be used safely. To ensure this quality, analysis by means of reliable methods is essential. An important point hereby, is the purity control of drug substances or drug products. As the main compound – the active pharmaceutical ingredient (API) – is synthesized through an organic synthesis pathway, the presence of impurities is inevitable. Besides the focus on the API and its assay, also the identification and quantification of impurities is often requested, for example for sample stability indicating methods. According to the guidelines of the International Conference of Harmonization (ICH), identification of impurities below the 0.05% level is considered to be unnecessary unless they are expected to be toxic [13-15]. Since quantification is easier if the separation is better, methods with baseline separations are often wanted. Certainly when an impurity peak overlaps with an API peak, identification and reliable quantification are problematic.

In the case of mixture 2, the compound cortisone was selected and simulated as API. When analyzing the API adapted sample of mixture 2 on its original optimized SOSLC column (6 cm C30 connected to 9 cm C18 SH 2), a peak overlap of cortisone with prednisolone is observed, as well as with an unknown impurity from the cortisone standard (Figure 6.5A). When analyzing the API adapted sample of mixture 2 on the extended optimal SOSLC column, a baseline separation between cortisone and prednisolone was obtained (Figure 6.5B). Note again that multiplication of the column

length together with proportionally adjusting the gradient profile does not influence the chromatogram profile and selectivity.

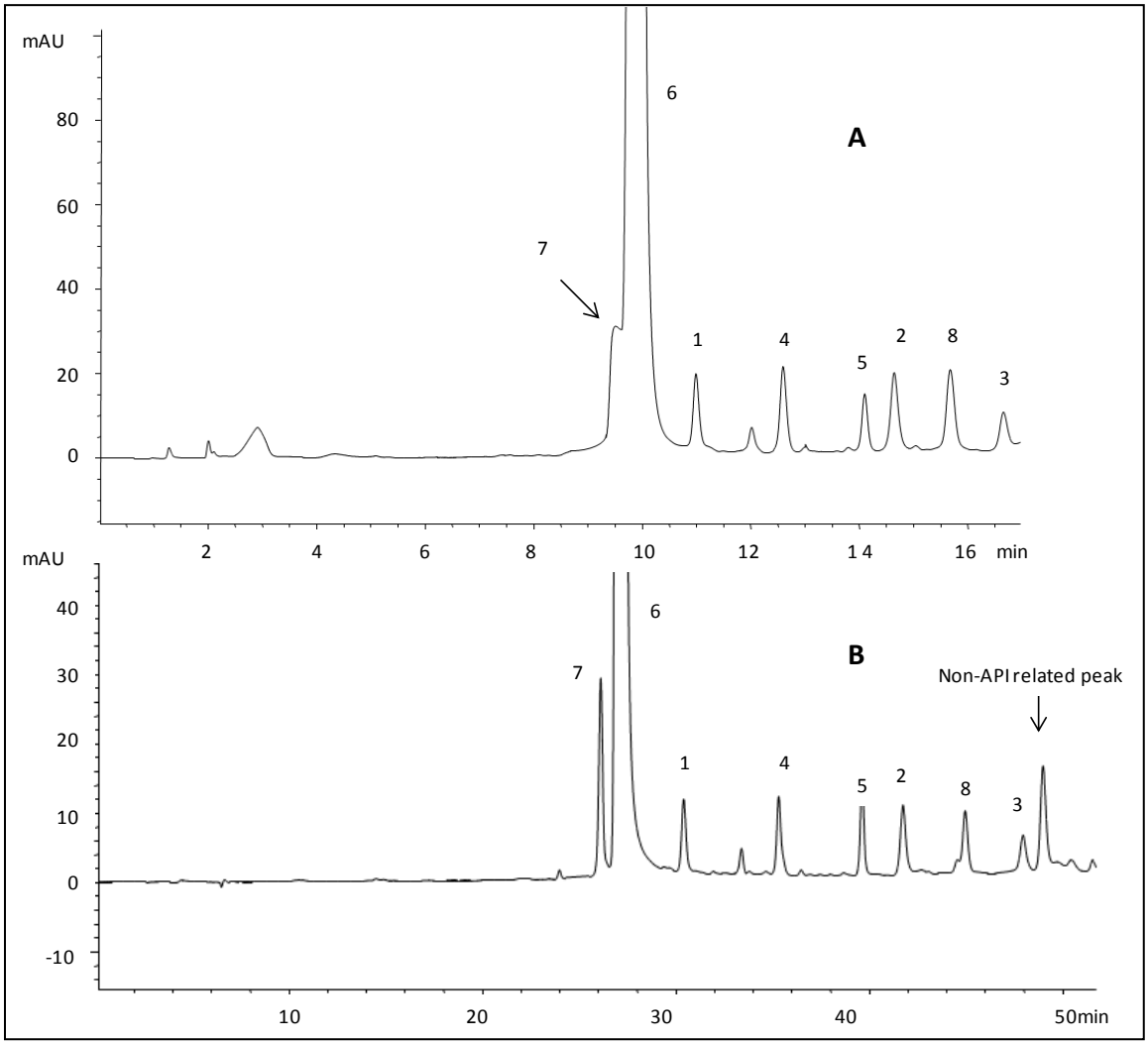

Fig. 6.5 A: Experimental chromatogram for the API (cortisone) simulated separation of mixture 2 on the optimal SOSLC column (6 cm C30 + 9 cm C18 SH2; total 15 cm). Gradient profile: water/ACN: 80/20 → 10/90 in 25 minutes. B: Experimental chromatogram for the API (cortisone) simulated separation on the extended optimal SOSLC column (18 cm C30 + 27 cm C18 SH2; total 45 cm). Gradient profile: water/ACN: 80/20 → 10/90 in 75 minutes. See table 6.2 for peak identification.

Also for mixture 1 an API analysis was simulated. Atrazin-desethyl was hereby selected as API simulated compound. On the original optimized SOSLC column, aldicarb is then the neighboring impurity peak. Again, analysis on this 25 cm SOSLC column shows overlap between atrazin-desethyl and aldicarb due to the broad band of atrazin-desethyl (Figure 6.6A). Identification of aldicarb is critical and quantification is impossible. Separation on the extended SOSLC column (4 cm C30, followed by 34 cm C18 EPS2 and ending with 12 cm C18 SH2), results in a better resolution, allowing a more obvious detection of aldicarb at a minimum normalized presence of 0.05% area (Figure 6.6B).

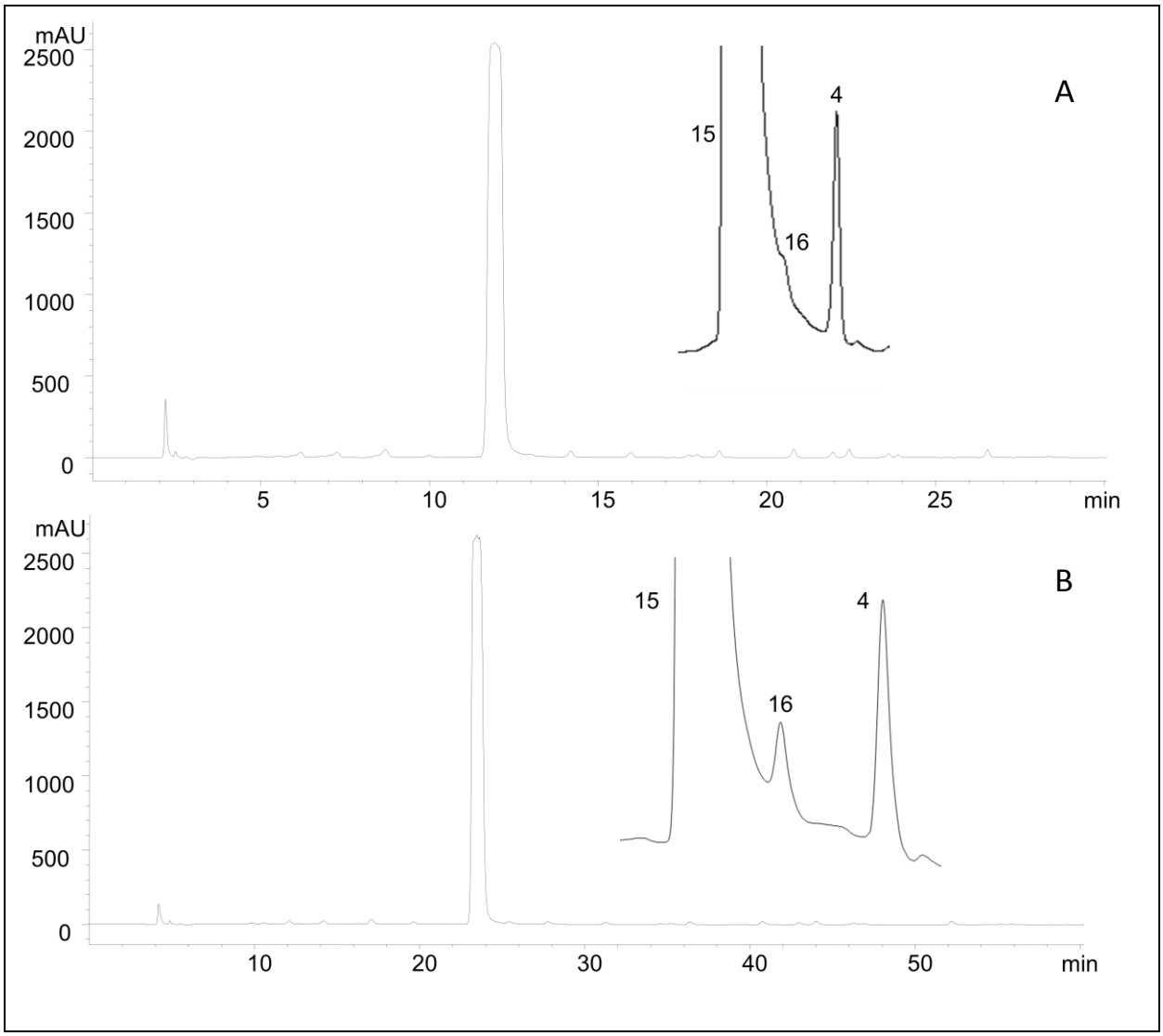

Fig. 6.6 A: Experimental chromatogram for the API (atrazin-desethyl) simulated separation of mixture 1 on the optimal SOSLC column (2 cm C30 + 17 cm C18 EPS2 + 6 cm C18 SH2; total 25 cm). Gradient profile: water(0.1% FA)/MeOH: 80/20 → 10/90 in 30 minutes. B: Experimental chromatogram for the API simulated separation on the extended optimal SOSLC column (4 cm C30 + 34 cm C18 EPS2 + 12 cm C18 SH2; total 50 cm). Gradient profile: water(0.1% FA)/MeOH: 80/20 → 10/90 in 60 minutes. See Table 6.1 for peak identification.

In general, the separation of two peaks can be assessed by the resolution R_S. In the case of separating an API from an impurity, the tailing of the API peak caused by the overload of the API on a column can affect the separation in such a way that no baseline separation with the impurity is obtained as the impurity will elute in the shoulder of the broad API peak. In this case, the discrimination factor d_0 can be used as a parameter to define the degree of separation [16]. This discrimination factor is used to assess the difficulty of accurate integration of a shoulder peak and is calculated as follows:

$$d_0 = \frac{h_p - h_v}{h_v} \qquad \text{(Eq. 6.1)}$$

where h_p is the height of the impurity peak and h_v the height of the valley by drop as shown in Figure 6.7 for the increased efficiency separation in Fig. 6.6B. From equation 6.1, it is clear that the lower h_v, the higher the value of d_0 will be.

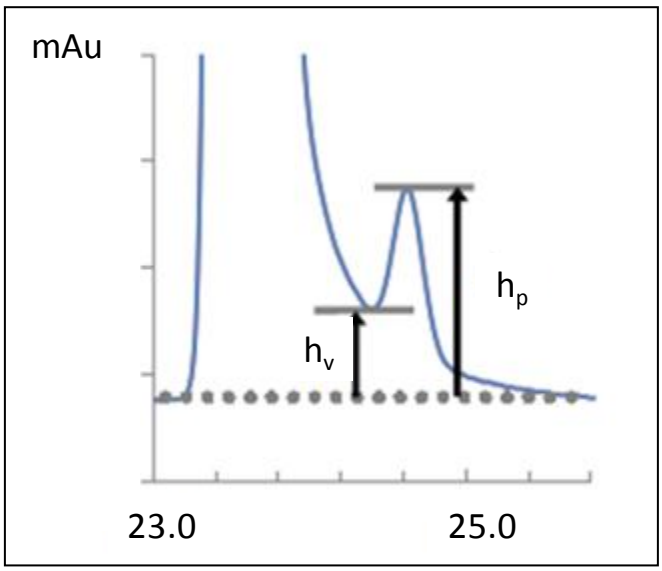

Fig. 6.7 The assessment of h_p and h_v with the separation of peak 15 and 16 in the chromatogram shown in Fig. 6.6B.

This results in a discrimination factor of 0.62 for the separation of peaks 15 and 16. Concerning the discrimination factor, a value of 0.5 can be sufficient for early phase, but for a late-stage phase, a value of 0.8 is recommended if the method will be validated for quantitative usage [17]. In the chromatogram of Fig. 6.6A, a value near 0 would be obtained for the discrimination factor. These results illustrate the benefit of increased efficiency in combination with SOSLC.

6.4 Conclusion

SOSLC is a selectivity optimization technique whereby the stationary phase is optimized by means of column segments of different stationary phases. Acceptable selectivity is calculated hereby. For mixtures containing multiple critical peak pairs or for mixtures containing a compound with a high concentration such as an API in relation to other compounds or impurities, it may happen that an optimized SOSLC column with classic dimensions, and in general a conventional HPLC column, does not offer sufficient resolution. Extending the column length of a conventional HPLC column with one stationary phase increases the efficiency and can offer sometimes a solution. However, if the separation problem remains elusive with insufficient selectivity, then the combination of SOSLC with column prolongation, offers a broader resolution space for obtaining a more sufficient separation in general, as well as separating a mixture with an intense broad peak or API peak.

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General conclusions and future perspectives

In the introduction of this thesis (chapter 1), the role of HPLC with the focus on RP-LC is outlined. Often selective separations are required making selectivity a primary target during method development. In chapter 2, conventional parameters which are typically used for fine-tuning and improving the selectivity of a RP-LC separation are described through their definition, a fundamental elucidation and how they can be modeled. These parameters include the solvent type, the solvent strength for isocratic analysis, the gradient profile for gradient analysis, the pH and the temperature. All these parameters can be screened in scouting runs in such a way that variations between these runs incorporate the feasible limits of each parameter. Such scouting runs can sometimes already offer a fit-for-purpose selective separation. If this is not the case, experimental design can be applied to search for optimal or fit-for-purpose separation conditions. Then the question raises which and how many parameters will be part of the experimental design. The more parameters taken into account, the larger the solution space will become, but the more complex the design and the modeling will be with a direct influence on the level of accurate prediction of these optimal conditions. So hereby, a personal choice or strategy can raise here towards the considered parameters.

Further on in this thesis, the possibilities of HPLC method development were expanded, whereby improved exploitation of the possibilities offered through the combination of stationary phases is explored in detail. Although HPLC is used by over several hundreds of thousands of users worldwide, surprisingly, the stationary phase is often not considered as a selectivity tunable parameter for a separation. This is mainly a consequence of the reluctance of routine environments, whereby validated and transferable methods are used. Coupling columns is assumed to detrimentally affecting the methods' robustness. Nevertheless, the author hopes that the illustration of the power of combining stationary phase segments of varying length to generate the best possible separation of a mixture of compounds, convinces future HPLC users that the development of a method whereby all peaks are maximally separated is inherently more or even at least as robust compared to many single column methods, often depicting incomplete separation when small changes in the environment are occurring. Besides the robustness issue, this perspective can be justified by the tendency that methods are nowadays more and more divided between in-house or single-laboratory methods which should be fit-for-purpose and compendial or multi-laboratory methods. For example, if for a temporarily in-house R&D purpose a method with a defined analytical target profile is required within a short time, SOSLC can be a powerful selectivity optimization tool beyond the typical

optimization approaches which are conservatively maintained in industries such as, for example, the pharmaceutical industry. Separation problems which remains elusive with a single column or stationary phase could be tackled by considering SOSLC as an optimization strategy. Nowadays, SOSLC has yet to be discovered by the industries which apply HPLC to a large extend. However, method developments strategies which recognize the benefits of orthogonal stationary phases do exist. But the next step, where combined tailor made SOSLC columns for complex separation problems are obtained, stays behind. The occurrence of a total error on the predictions compared to the experimental retention times or selectivity factors in the SOSLC approach is evident. Premising a null hypothesis which strives for no error on the predictions is surpassed as the origin of systematic errors and random errors is not easy to trace. The applied retention model, typically quadratic, and the mathematical prediction algorithm can both lead to errors besides typical instrumental sources of variation. In order to evaluate and to handle errors on the predictions, it might be preferable to work with acceptance limits on the prediction residuals. With this philosophy, the origin of errors become less important as long as the prediction approach is fit-for-purpose.

Summarized, the strategy of SOSLC is investigated and extended to several LC operational modes such as multi-step gradient (chapter 3), linear gradient (chapter 4), green chromatography (Chapter 5), multi-linear gradients (chapter 5) and increased efficiency LC (chapter 6). In the margin of the research targets and content described in these chapters, other research targets could be considered for further investigation or consideration.

The same set of stationary phases has been applied for the different research objectives in this thesis. Based on their chemistry, they are with reason considered to be orthogonal and therefore suitable for SOSLC. But with the more than thousand existing commercial RP-LC columns on the market and with the almost daily addition of new columns, it might be a challenge to look for the utmost set of orthogonal stationary phases within all these available phases. The existing available column classification approaches might thereby serve as a useful starting impulse.

SOS-LC as it is currently available and applicable, nevertheless, has also been explored by others since the start of writing of this thesis and its finalization.

SOS-LC has been applied as technique for the analysis of drugs in more complex matrices such as human autopsy material [1]. Furthermore, the implementation of mobile phase composition optimization and gradient profile optimization within SOS-LC has been reported in the literature [2-5].

Using conventional columns and serially coupling them with commercially available fittings and tubing instead of using the manufactured POPLINK column segments has also been investigated in the laboratory and has been confirmed as a feasible option [6].

Even in the field of chip-based chromatography, the selectivity strategy of combining different stationary phases has been investigated with the term phase optimized chip-based liquid chromatography [7].

With perspective to the separation of enantiomers, the serially coupling of achiral and chiral columns have been investigated as well [8].

Also, by using the same stationary phases and column segments for this work, another perspective remains to expand and investigate the power of SOSLC with column segments packed with sub-2µ particles which are the base of Ultra High Pressure Liquid Chromatography (UHPLC). UHPLC leads to a higher efficiency and offers the possibility to speed up analyses without the loss of resolution.

Considering the theoretical part of constructing retention models, the use of preliminary gradient runs instead of isocratic runs could be desirable in some cases. A possible concept, using a genetic algorithm or a monte carlo simulation, could consist of the generation of modeling coefficients and their implementation in the prediction algorithm described in chapter 3. Verifying the simulated gradient retention times with the preliminary obtained scouting retention times could lead to acceptable approximation of the modeling coefficients.

Even considering the chromatographic separation mode, SOSLC can be expanded beyond the use of RP-LC. It's usage could be investigated in e.g. HILIC mode whereby nowadays multiple stationary phases are available as well. Another route is the implementation of SOSLC in SFC, resulting in SOSSFC [9], as SFC gains an increasing interest for several applications such as the separation of chiral compounds.

In general, it can be concluded that SOSLC is a now 21st century tool at its starters' stage in the field of chromatography and offers a lot of perspectives towards plenty other expansion ideas or towards combination with other innovative tendencies. Nevertheless, the author believes in a conjuncture, even in the field of chromatography. Topics raise, they are investigated by many researchers for several years, and then they go. This is illustrated by the countless amount of papers about mobile phase and gradient optimization in the eighties and nineties. Once it was believed that everything was investigated and clarified at that stage, other topics raised. The last decade a lot of research has been performed with the focus on efficiency. It wouldn't be surprising that selectivity will return as a hot topic for the next years due to the new ideas and the current understandings in chromatography

which are incontrovertibly achieved by the research performed in other topics such as the profound investigation of efficiency, column packings, elevated temperature, etc..

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Summary

The aim of this thesis was to evaluate if the stationary phase could be a potential tool and adjustable parameter for improved selectivity optimization beyond classic approaches in Reversed Phase Liquid Chromatography (RP-LC). Today RP-LC is an essential analysis and separation technique in a variety of industries (e.g. the pharmaceutical industry). Innovative ideas in which the stationary phase can play such a role of a systematic adjustable parameter for selectivity optimization (Stationary Phase Optimized Selectivity Liquid Chromatography, SOSLC) are worked out and investigated in this work.

In chapter 2, the fundamentals and principles of nowadays selectivity optimization in High Performance Liquid Chromatography (HPLC) are first reviewed. Hereby, the relevant fundamental chromatographic terms are explained. The importance of the conventional parameters such as solvent type, solvent strength, pH and temperature are outlined. Further on, the link towards the stationary phase as optimization parameter is made and serves as a prologue for the work described in Chapters 3, 4, 5 and 6.

Stationary phase optimized selectivity liquid chromatography (SOSLC) is an approach to tune a given LC separation by combining different stationary phases in a multi-segment column set-up. SOSLC and its optimization procedure and algorithm were originally introduced, while only being applicable to isocratic conditions, which is a severe limitation for the analysis of mixtures composed of components covering a broad hydrophobicity range. A strategy is therefore described in chapter 3 to circumvent this limitation. The components of a mixture are thereby divided into different groups according to hydrophobicity as elucidated by a preliminary gradient analysis on a C18 reversed-phase column. Each group separation is then individually optimized with a specific isocratic mobile phase composition using the original isocratic SOSLC strategy. The mobile phase composition thereby only differs in the percentage of organic modifier between the various groups. Finally, a combination of stationary phases that guarantees sufficient selectivity for all the groups is selected and the separation is performed by a multiple step gradient, whereby each level consists of the mobile phase composition applied for the SOSLC optimization of the individual groups. The multiple step gradient approach is demonstrated through the analysis of a mixture of 27 steroids covering a wide range of hydrophobicity.

Step gradient SOSLC has been developed and described in chapter 3 but this is not elegant for the analysis of complex mixtures composed of components covering a broad hydrophobicity range as exact retention times are not predicted. A linear gradient prediction algorithm was therefore

subsequently developed allowing to apply SOSLC as a generic RP-LC optimization method and is described in chapter 4. Hereby, a gradient – independent of its shape and steepness – is considered as a sequence of multiple small isocratic levels. For each isocratic level, the migration distance and the migration time of the analyte band is monitored. Numeric integration of these intermediate migration times – subjected to the necessary corrections – leads to a prediction of retention time under gradient conditions. The algorithm allows operation in isocratic, step-wise and linear gradient run modes. The features of SOSLC in linear gradient mode are demonstrated by means of a mixture of 13 steroids, whereby baseline separation is predicted and experimentally demonstrated.

Chapters 3 and 4 have shown that SOSLC offers excellent possibilities for method development, especially after the modification towards linear gradient SOSLC. The content of chapter 5 is aimed at developing and extending the SOSLC approach towards selectivity optimization and method development for green chromatography application. Contrary to current LC practices, a green mobile phase (water/ethanol/formic acid) is hereby preselected and the composition of the stationary phase is optimized under a given gradient profile to obtain baseline resolution of all target solutes in the shortest possible analysis time. The principle is illustrated with a fast, full baseline resolution for a randomly selected mixture composed of sulphonamides, xanthine alkaloids and steroids.

Depending on the sample to be analyzed, an SOSLC optimized separation method can be obtained under multiple run modes such as isocratic, step-wise gradient and linear gradient mode which are described in chapter 3, chapter 4 and chapter 5. The fundamentals and features of SOSLC have been described and demonstrated in these previous chapters. In the field of pharmaceutical analysis, selectivity is an important issue for separating impurities from the active pharmaceutical compound (API), which is typically characterized by a broad and concentrated peak. Overlap with an impurity is an often confronted problem when a separation method needs to be developed. In chapter 6, the benefits of the SOSLC approach for optimizing the selectivity in combination with the use of extended column lengths for increasing the efficiency are described. This combination of selectivity and increased efficiency leads to an improved resolution. This is demonstrated by means of representative test mixtures whereby API and impurity simulation is performed. Evaluation is performed by means of the discrimination factor as a quality criterion.

Samenvatting

Het doel van deze thesis – beschreven in Hoofdstuk 1 – was na te gaan of de stationaire fase een potentiële rol kan spelen of een toegevoegde waarde kan hebben als een optimaliseerbare parameter voor selectiviteitsoptimalisatie in vergelijking met de typische optimalisatiestrategiën in omkeerfase vloeistofchromatografie. Omkeerfase vloeistofchromatografie is een belangrijke scheidings- en analysetechniek in meerdere industrieën zoals bijvoorbeeld de farmaceutische industrie. Innovatieve ideeën waarin de stationaire fase een rol kan spelen als een systematische optimaliseerbare parameter voor selectiviteit (*Stationary Phase Optimized Selectivity Liquid Chromatography*, SOSLC) zijn uitgewerkt en onderzocht in deze thesis.

In Hoofdstuk 2 zijn de principes en de fundamentele achtergronden van selectiviteitsoptimalisatie in hoge druk vloeistofchromatografie beschreven. Hierin worden de termen en basisnoties van chromatografie toegelicht.

SOSLC laat toe een vloeistofchromatografie scheiding te beïnvloeden door het combineren van verschillende stationaire fasen in een multi-segment kolom. SOSLC werd geïntroduceerd als een strategie in de wereld van chromatografie waarbij de optimalisatie procedure en het predictie algoritme enkel van toepassing waren op isocratische scheidingen. Dit is een beperking voor de scheiding van complexe mengsels met analieten over een wijd bereik van hydrofobiciteit. Hiervoor is een strategie beschreven in Hoofdstuk 3, die deze beperking van isocratische analyse omzeilt. De componenten van een mengsel worden onderverdeeld in verschillende groepen volgens hun relatieve hydrofobiciteit, dewelke getoetst wordt door een generieke gradient analyse op een octadecyl silica kolom. Elke groep wordt dan apart geoptimaliseerd volgens de originele isocratische SOSLC optimalisatiestrategie waarbij voor elke groep een passende mobiele fase verhouding gebruikt wordt. Deze verhoudingen verschillen dus onderling – voor elke groep – in het geselecteerde percentage organische modifier. Uiteindelijk wordt gezocht naar een stationaire fase combinatie die voldoende selectiviteit garandeert voor alle groepen. Hierbij wordt een multiple step gradient aangewend die bestaat uit de consecutieve opvolging van de individueel geselecteerde mobiele fase verhoudingen. Deze aanpak is geïllustreerd met de scheiding van 27 steroïdale componenten.

De strategie beschreven in Hoofdstuk 3 heeft zijn toegevoegde waarde maar is misschien niet de meest elegante oplossing voor de scheiding van een complex mengsel omdat de exacte retentietijden niet voorspeld worden. In hoofdstuk 4 wordt de ontwikkeling van een algoritme besproken dat de predictie toelaat van retentietijden onder gradient condities en toegepast op de

techniek SOSLC. De sleutel hierbij is dat een gradient beschouwd wordt als de opvolging van meerdere kleine isocratische stappen. Numerieke integratie van deze kleine stappen vertaalt zich dan in een gradient en bijgevolg in een predictie van retentietijden. SOSLC voor lineaire gradienten is geïllustreerd aan de hand van een mengsel van 13 steroiden waarbij basislijnscheiding bekomen wordt.

Hoofdstukken 3 en 4 tonen het nut aan van SOSLC als middel in methode-ontwikkeling. Hoofdstuk 5 focust op de implementatie van SOSLC in het topic van groene chromatografie. Toxische solventen worden vervangen door groene solventen en scheidend vermogen – voorheen behaald door de unieke eigenschappen van deze toxische solventen – wordt bekomen door toepassing van SOSLC. Wederom is het opzet van dit hoofdstuk geïllustreerd aan de hand van een modelmengsel.

In de farmaceutische industrie wordt selectiviteit belangrijk als onzuiverheden gescheiden dienen te worden zowel onderling als van het actief farmaceutisch ingrediënt. Het actief farmaceutisch ingrediënt is typisch gekenmerkt door een grote brede piek. Hierbij is overlap met een onzuiverheid geen zeldzaam voorval. In hoofdstuk 6 wordt onderzocht of de aanpak van SOSLC kan gecombineerd worden met verlengde kolomlengtes en dus verhoogde efficiëntie. De combinatie van beide aanpakken zou moeten leiden tot verbeterde resolutie van kritische piekparen. Dit idee is toegepast op twee testmengsels. Evaluatie van de scheiding van een API met een aangrenzende onzuiverheid is gebeurd door de discriminatie factor.

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Abbreviations

AA	Acetic acid
ACC	Automated Column Coupler
ACN	Acetonitrile
API	Active Pharmaceutical Ingredient
CN	Cyanopropyl
DP	Drug product
DS	Drug substance
EPS	Enhanced Polar Selectivity
ET-LC	Elevated Temperature Liquid Chromatography
EtOH	Ethanol
FA	Formic acid
Fig.	Figure
HETP	Height Equivalent of a Theoretical Plate
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Performance Liquid Chromatography
HSM	Hydrophobic Subtraction model
ICH	International Conference on Harmonization
ID	Internal diameter
IEC	Ion Exchange Chromatography
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid Chromatography
LSS	Linear Solvent Strength
MeOH	Methanol
MS	Mass Spectrometry
NIST	National Institute of Standards and Technology
NP-LC	Normal Phase Liquid Chromatography
ODS	Octadecyl silica
OTLC	Open Tubular Column
PCA	Principal Component Analysis
PCB	Polychlorinated biphenyls
PEEK	Polyether ether ketone
PH	Phenyl
POPLC	Phase Optimized Selectivity Liquid Chromatography
PQRI	Product Quality Research Institute
QSAR	Quantitative Structure-Activity Relationship
QSRR	Quantitative Structure-Retention Relationship
RP-LC	Reversed Phase Liquid Chromatography
SAX	Strong Anion Exchange
SCX	Strong Cation Exchange
SEC	Size Exclusion Chromatography
SOSLC	Stationary Phase Optimized Selectivity Liquid Chromatography
SRM	Standard Reference Material
Temp.	Temperature
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography

USP	United States Pharmacopeial Convention
UV-VIS	Ultraviolet - visible
VBA	Visual Basic
VL-MD	Variable column length Method Development
WAX	Weak Anion Exchange
WCX	Weak Cation Exchange

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Acknowledgements

Appendix A: List of coefficients, symbols and terms

Δt: a time interval obtained by Eq. 4.7.

Δt_i: a time interval during the dwell time of a linear gradient analysis obtained by Eq. 4.7.

Δt_j: a time interval after the dwell time of the linear gradient analysis has passed obtained by Eq. 4.7.

v_0: the linear velocity of the mobile phase.

Φ_A: the length of a column segment, packed with stationary phase A, present in a combined column segment SOSLC column containing multiple stationary phases.

φ: the percentage of the organic modifier concentration in the mobile phase.

φ_0: the initial percentage of the organic modifier concentration in the mobile phase in a gradient program described according Eq. 4.4.

a, b and *c*: the experimental determined regression coefficients for obtaining Eq. 4.1 and Eq. 4.2.

d_c: the covered distance of a compound through a column during a time interval Δt.

$d_{c,\Delta t_i}$: the covered distance of a compound through a column during a time interval Δt_i during the dwell time.

$d_{c,\Delta t_j}$: the covered distance of a compound through a column during a time interval Δt_j after the dwell time of the linear gradient analysis has passed.

$d_{c,dwell}$: the total covered distance of a compound through the column at the moment the dwell time has passed.

$d_{c,int}$: the intermediary covered distance of a compound through a column at a certain moment, $t_{r,int}$, of the analysis.

k: the retention factor of a compound.

k_A: the retention factor of a compound on stationary phase A.

L: the column length used during the preliminary measurements.

p: a settable parameter in order to vary the width of Δt.

q: the slope of the linear gradient profile described according Eq. 4.4.

t: time.

t_0: the void time.

t_{dwell}: the system dwell time.

$t_{dwell,column}$: the dwell time contribution occurring in the column.

$t_{dwell,column,\Delta t_j}$: the increased dwell time in the column at Δt_j.

t_t: the retention time of a compound as it would be for an isocratic analysis on a column with the length used for the preliminary measurements; t_t is used in intermediate calculations.

$t_{r,dwell,int}$: the intermediary passed analysis time during the dwell time of the analysis.

t_r: calculated retention time of a compound for a linear gradient analysis.

$t_{r,int}$: the intermediary passed analysis time when a compound covered a distance $d_{c,int}$ through the column.

Appendix B: VBA script for prediction of gradient retention times by the algorithm described in Chapter 4

In appendix B, a programmed script in Visual Basic as a part of Microsoft Excel is presented which allows the calculation of the retention time of a component under linear gradient conditions. In this example script, the possibility to predict a retention time for a multi-linear gradient profile with one node is integrated as well.

```
Sub Macro1()

' macro 1 comp

'lnk
Dim lnk As Single
Dim k As Single

'regression coefficients' lnk = a phi³ + b phi² + c phi + d
Dim a As Single
Dim b As Single
Dim c As Single
Dim d As Single
Dim phi As Single

Dim a2 As Single
Dim b2 As Single
Dim c2 As Single
Dim d2 As Single

'dwell time
Dim td As Single
Dim tdsec As Single

td = Cells(63, 4)
tdsec = td * 60
Cells(64, 4) = tdsec

'lowest void time
Dim tm As Single
Dim tmsec As Single

tm = Cells(62, 4)
tmsec = tm * 60
Cells(61, 4) = tmsec

'gradient profile
Dim aa As Single
Dim bb As Single
```

```
'column length
Dim cl As Single
cl = Cells(63, 9)

'retentietijd tr
Dim tr As Single

'instant covered distance (cm/sec)
Dim cd As Single

'intermediate/total covered distance (cm)
Dim cdtot As Single
Dim cdtot2 As Single

'final retention time
Dim trfin As Single

'mobile phase speed
Dim v As Single
v = cl / tmsec

'counter
Dim counter As Long

'round cdtot/v
Dim cdtotv As Single
Dim roundcdtotva As Single
Dim roundcdtotvb As Single
Dim roundcdtotvc As Single

'comp 1
a = Cells(43, 4)
b = Cells(43, 5)
c = Cells(43, 6)
d = Cells(43, 7)

'comp 2    3th degree polyn is better then 2nd degree!!!!!! To do: put decision option in program!!
a2 = Cells(43, 8)
b2 = Cells(43, 9)
c2 = Cells(43, 10)
d2 = Cells(43, 11)

'COMP 1 CALCULATIONS

cdtot = 0
cdtot2 = 0

'start dwell time part + ctu increasing dwell time during dwell time

counter = 1
For counter = 1 To (tdsec + roundcdtotv) Step 1
```

```
    phi = Cells(67, 6)
    lnk = (a * (phi ^ 3)) + (b * (phi ^ 2)) + (c * phi) + d
    k = Exp(lnk)
    tr = (k * tmsec) + tmsec
    cd = cl / tr
    cdtot = cdtot2 + cd

    cdtot2 = cdtot

    'afronden cdtot/v
    cdtotv = cdtot / v
    roundcdtotva = Round(cdtotv, 0)
    Cells(39, 1) = roundcdtotva

    If cdtot > cl Then
       Cells(77, 2) = counter / 60
       Cells(77, 3) = cdtot
       Exit For
    End If

Next counter

'(physically) gradient part 2 ( = 1st part of the gradient profile which starts after the dwell time)

counter = tdsec + 1 + roundcdtotva

'note: roundcdtotv addes as term!!! Important to know phi around the migrating analyte band!!

For counter = (tdsec + roundcdtotva + 1) To (Cells(68, 8) + tdsec + roundcdtotvb) Step 1

    'regression values gradient profile phi = at + b   (remark: t in sec!)
    'rico (a)
    aa = (Cells(68, 6) - Cells(67, 6)) / (Cells(68, 8) - Cells(67, 8))
    'b
    bb = Cells(68, 6) - (aa * Cells(68, 8))

    phi = bb + (aa * (counter - tdsec - (cdtot / v)))

       If phi < Cells(67, 6) Then
       phi = Cells(67, 6)
       End If

    lnk = (a * (phi ^ 3)) + (b * (phi ^ 2)) + (c * phi) + d
    k = Exp(lnk)
    tr = (k * tmsec) + tmsec
    cd = cl / tr
    cdtot = cdtot2 + cd

    cdtot2 = cdtot

    'afronden cdtot/v
```

```
   cdtotv = cdtot / v
   roundcdtotvb = Round(cdtotv, 0)
   Cells(40, 1) = roundcdtotvb

   If cdtot > cl Then
      Cells(78, 2) = counter / 60
      Cells(78, 3) = cdtot
      Exit For
   End If

Next counter

'(physically) gradient part 3 (= 2nd part of the gradient profile)

For counter = (Cells(68, 8) + tdsec + roundcdtotvb + 1) To (Cells(69, 8) + tdsec + roundcdtotvc) Step 1

   'regression values gradient profile phi = at + b   (remark: t in sec!)
   'rico (a)
   aa2 = (Cells(69, 6) - Cells(68, 6)) / (Cells(69, 8) - Cells(68, 8))
   'b
   bb2 = Cells(69, 6) - (aa2 * Cells(69, 8))

   phi = bb2 + (aa2 * (counter - tdsec - (cdtot / v)))

      If phi < Cells(68, 6) And (counter - tdsec - (cdtot / v)) < tdsec Then
      phi = Cells(67, 6)
      End If

      If phi < Cells(68, 6) And (counter - tdsec - (cdtot / v)) > tdsec Then
      phi = bb + (aa * (counter - tdsec - (cdtot / v)))
      End If

   lnk = (a * (phi ^ 3)) + (b * (phi ^ 2)) + (c * phi) + d
   k = Exp(lnk)
   tr = (k * tmsec) + tmsec
   cd = cl / tr
   cdtot = cdtot2 + cd

   cdtot2 = cdtot

   'afronden cdtot/v
   cdtotv = cdtot / v
   roundcdtotvc = Round(cdtotv, 0)
   Cells(41, 1) = roundcdtotvc

   If cdtot > cl Then
      Cells(79, 2) = counter / 60
      Cells(79, 3) = cdtot
      Exit For
   End If

Next counter
```

End Sub